Active and Adoptive Immunotherapy in Indolent Lymphoproliferative Diseases

Thesis

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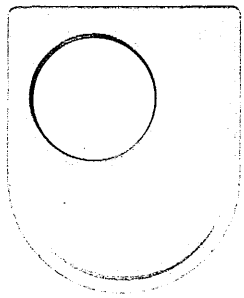

The Open University

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ABSTRACT

Although monoclonal antibody (mAb) therapy has improved the outcome of low-grade B-cell non-Hodgkin lymphomas (B-NHL), they remain incurable diseases. The intimate relationship between immune cells and lymphomas for its maintenance and progression suggests that immunotherapy may represent a valuable strategy towards the control and eradication of these malignancies. Despite initial successes of anti-idiotype vaccination, its clinical benefits have not been definitely proven. New insights into the mechanisms whereby (i) tumour escape immunity, (ii) certain anticancer treatments exert immunogenic functions, (iii) microenvironment influences lymphoma growth should open new ways for immunotherapeutic intervention.

Towards this goal, dendritic cells (DCs) loaded with autologous killed tumour cells were used to immunize indolent B-NHL patients against a wide spectrum of tumour antigens, thereby enhancing the possibility of a clinical success. A significant correlation was observed between clinical responses and both regulatory T cell frequency decrease and natural killer cell activation. These effects were positively associated with calreticulin and heat shock protein (HSP)90 surface expression in dying tumour cells used in the vaccine formulations. Therapeutic improvements might thus be accomplished by stimulating tumour release of "eat-me" signals.

T-cell maturation and anti-lymphoma activation were also associated with a positive clinical outcome. A novel artificial expansion system was developed to boost these effects through the *ex-vivo* generation of functional, long-lasting polyclonal T cells and adoptive cell therapy.

Finally, the investigation of tumour-restricted humoral immunity in clinical responders permitted the serological identification of HSP105 as a novel potential NHL immunodominant antigen. HSP105 expression correlated with B-NHL proliferation and aggressiveness. A specific neutralizing Ab significantly reduced lymphoma burden in xenotransplanted mice, thus laying the basis for a novel passive immunotherapy for B-NHL.

Collectively, these results provide important information on mechanisms underlying anti-lymphoma immunity and open up the possibility of improving active and passive immunotherapy to the treatment of indolent B-NHLs.

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ABBREVIATIONS

5-FU	5-fluorouracile
7-AAD	7-Aminoactinomycin D
aAPCs	artificial antigen presenting cells
Abs	antibodies
ACT	adoptive cell transfer
ADCC	Ab-dependent cell cytotoxicity
AFP	alphafetoprotein
AIDS	acquired immune deficiency syndrome
ANN V	annexin V
APC	allophycocyanin
APCs	antigen presenting cells
ATP	adenosine triphosphate
BCA	bicinchoninic acid
BCR	B-cell receptor
BiTE	Bispecific T-cell engager
BL	Burkitt's lymphoma
BM	bone marrow
CDC	Complement-dependent cytotoxicity
CDR	complementarity determining region
CEA	carcinoembryonic antigen
CFSE	carboxyfluorescein diacetate succinimidyl ester
CHOP	cyclophosphamide, doxorubicin / hydroxydoxorubicin, vincristine and prednisone
CLL	Chronic lymphocytic leukemia
CML	chronic myeloid leukemia
CPT-11	irinotecan
CR	complete responder
CRT	calreticulin
CTB	cholera toxin B
CTL	cytotoxic T lymphocytes
CTLA-4	cytotoxic T lymphocytes-associated antigen 4
CTRL	control
CVP	cyclophosphamide, vincristine, and prednisone
DCs	dendritic cells
DLBCL	diffuse large B-cell lymphoma
DLI	donor lymphocyte infusion
DTH	delayed-type hypersensitivity
DXR	doxorubicin
EBV	Ebstein-Barr virus
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunosorbent spot
FACS	fluorescence-activated cell sorting
Fc	fragment crystallizable
FDA	food and drug administration
FITC	fluorescein isothiocyanate
FL	follicular lymphoma
FLIPI	follicular lymphoma prognostic index
FR	framework region
GC	germinal centre
GIST	gastrointestinal stromal tumour

GITR	glucocorticoid-induced TNF receptor family-related gene
GM-CSF	granulocytes-macrophages colony stimulating factor
GMP	good manufacturing practice
GVHD	graft-versus-host disease
GVL	graft-versus lymphoma
HDS	high dose sequential chemotherapy
HER2	human epidermal growth factor receptor 2
HIV	human immunodeficiency virus
HLA	human leukocyte antigen system
HMGB1	high-mobility group box 1
HSCT	haemopoietic stem cell transplantation
HSP	Heat shock protein
i.p.	intraperitoneal
IC50	half inhibitory concentration
Id	Idiotype
IDO	indoleamine 2,3-dioxygenase
IFN-γ	interferon-γ
IFRT	involved-field radiation therapy
Ig	immunoglobulin
IHC	immunohistochemistry
IL	interleukin
IP	immunoprecipitation
IPG	Immobilized pH gradient
KEGG	Kyoto encyclopedia of genes and genomes
KLH	keyhole limpet hemocyanin
LAM	lymphoma-associated macrophage
LCL	lymphoblastoid cell line
LMPs	latent membrane proteins
LN	LN
LPL	lymphoplasmacytic lymphoma
LPS	lipopolysaccharide
M6P	mannose-6-phosphate
mAb	monoclonal antibody
MCL	mantle cell lymphoma
MD	microdomain
MDSC	myeloid-derived suppressor cells
MFI	mean/median fluorescence intensity
MHC	major histocompatibility complex
min	minutes
MNR	magnetic nuclear resonance
MRD	minimal residual disease
MS	mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MZL	marginal cell lymphoma
n	number
NA	neutravidin
NFC	nasopharyngeal carcinoma
NHL	non-Hodgkin lymphoma
NK	natural killer cells
NKT	natural killer T cells
NR	non-responder patient
NSCLC	non small cell lung cancer

OS	overall survival
p.I.	isoelectric point
PAP	prostate acid phosphatese
PBMCs	peripheral blood mononuclear cells
PD-1	Programmed Death-1
PD-L1	Programmed Death-1 ligand
PE	phycoerythrin
PerCP	peridinin chlorophyll protein
PFS	progression free survival
PHA	phytohaemagglutinin
PI	propidium iodide
PMA	phorbol 12-myristate 13-acetate
PR	partial responder
PSA	prostate-specific antigen
PSMA	prostate specific membrane antigen
PTLPD	post-transplant lymphoproliferative disease
R	responder patients
rh	recombinant human
RT	room temperature
s.c.	subcutaneous
SCID	severe combined immunodeficient
SDS-PAGE	sodium dodecyl sulphate - polyacrylamide gel electrophoresis
sec	seconds
SEREX	recombinant cDNA expression libraries
SERPA	serological proteome-based approach
SMIPs	small-modular immunopharmaceuticals
STAT	signal transducer and activator of transcription
STAT3	signal transducer and activator of transcription-3
STIR	short tau inversion recovery
TAA	Tumour-associated antigen
T_{CM}	central memory T cells
TCR	T-cell receptor
Teff	effector T cells
T_{EM}	effector memory T cells
TGF-β	tumour growth factor beta
Th	T helper
TILs	tumour-infiltrating lymphocytes
TLR	Toll like receptor
TNF	tumour necrosis factor
TNFR	tumour necrosis farctor receptor
Tregs	regulatory T cells
TSE	turbo-spin-echo
UVC	ultraviolet C rays
VEGF	vascular endothelial growth factor
vs.	versus
WB	western blot
WHO	world health organization
γ	gamma-irradiation

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1 INTRODUCTION

1.1 Indolent B-cell NHL

1.1.1 Clinical and biologic characteristics

Non-Hodgkin lymphomas (NHLs) are a group of malignancies that arise from mature T- or B-lymphocytes in the lymphoid tissue, which includes the lymph nodes, spleen, and other organs of the immune system. NHLs are the seventh most common cancer in males and females in the United States. In the Western world, about 45 new cases of lymphoma are diagnosed per 100,000 people per year (Fisher and Fisher 2004), with an even higher incidence in elderly people. The age-adjusted incidence of NHLs rose by more than 79% from 1975 to 2005, representing an average annual percentage increase of about 2.6 percent, one of the highest registered. The reasons for this increase are not certain, and there are probably multiple causes, including human immunodeficiency virus (HIV) infection or acquired immune deficiency syndrome (AIDS) and the massive introduction of herbicides and pesticides containing organochlorine, organophosphate and phenoxyacid, all compounds that are linked to lymphoma. Exposure to certain viruses and bacteria, such as Ebstein-Barr Virus (EBV) and Helicobacter Pylori, is associated with NHL. Furthermore, inherited syndromes, such as Sjögren and Klinefelter syndromes, can predispose individuals to the later development of NHLs. The concept of predisposition genes is under study to determine if they play a role in the sporadic occurrence of NHL in otherwise healthy individuals.

About 85% of NHLs are of B-cell origin (B-NHLs), the rest are T-cell or natural killer (NK) cell malignancies. Exciting progress has been made in the past 20 years to elucidate the cellular origin of human B-cell lymphomas and the identification of key transforming events, in particular the role of chromosomal translocations in lymphoma pathogenesis. B-cell development takes place in distinct differentiation steps that are characterized by the specific structure of the B-cell receptor (BCR). BCR development occurs via an error-prone process involving the combinatorial rearrangement of the V, D, and J gene segments in the heavy (H) chain locus and the V and J gene segments in the light (L) chain loci (Brack, Hirama et al. 1978) (Figure 1.1).

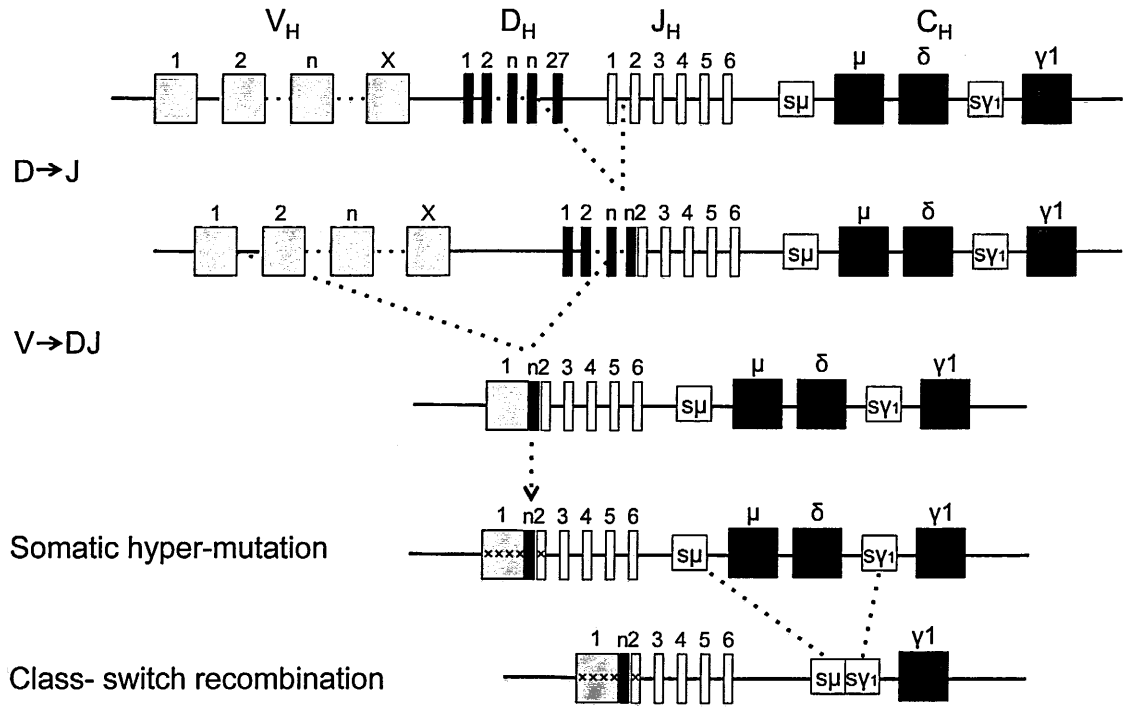


Figure 1.1 Molecular processes that remodel immunoglobulin genes.
Immunoglobulins (Igs) are expressed exclusively by B cells, after rearrangement of variable (V) regions, which interact with antigen, and constant (C) regions, which mediate their effector functions. (A) "V(D)J recombination" happen in the V regions of both heavy- (H) and light-chain (not shown) genes. About 50 functional VH gene segments, 27 DH segments and 6 JH segments are available in the germline, allowing the generation of a diverse repertoire of VH gene rearrangements. The diversity is further increased by the addition or removal of nucleotides at the joining sites of the gene segments. The process of somatic hyper-mutation is activated when B cells reach the germinal center (GC, shown in more details in Figure 5.2) and leads to the introduction of point mutations, deletions or duplications in the rearranged V-region of Ig genes ("X" in the Figure). Class switching leads to the replacement of the IgM (C μ (mu)) and IgD (C δ (delta)) C-region gene segments with the IgG (C γ (gamma)1) ones by recombination at the switch regions (S μ (mu) and S γ (gamma)1) and gives rise to an Ig with different effector functions but the same antigen-binding domain.

Mature (naïve) B cells carry a BCR composed of two identical heavy chain and two identical light-chain immunoglobulin (Ig) polypeptides that are covalently linked by disulphide bridges (Rajewsky 1996). Antigen recognition by naïve B cells favours their recruitment into secondary lymphoid follicles where they undergo somatic hypermutation of VH genes and class switch recombination at the IgH locus, and are selected for increased BCR-antigenic epitope affinity through affinity maturation. These processes form the germinal centre (GC) reaction that leads to the preferential expansion of the most suitable B cell clone(s) able to specifically clear pathogens and thus protect the host tissues (LeBien and Tedder 2008). Finally, GC B cells differentiate into memory B cells or plasma cells and leave the GC microenvironment (Figure 1.2).

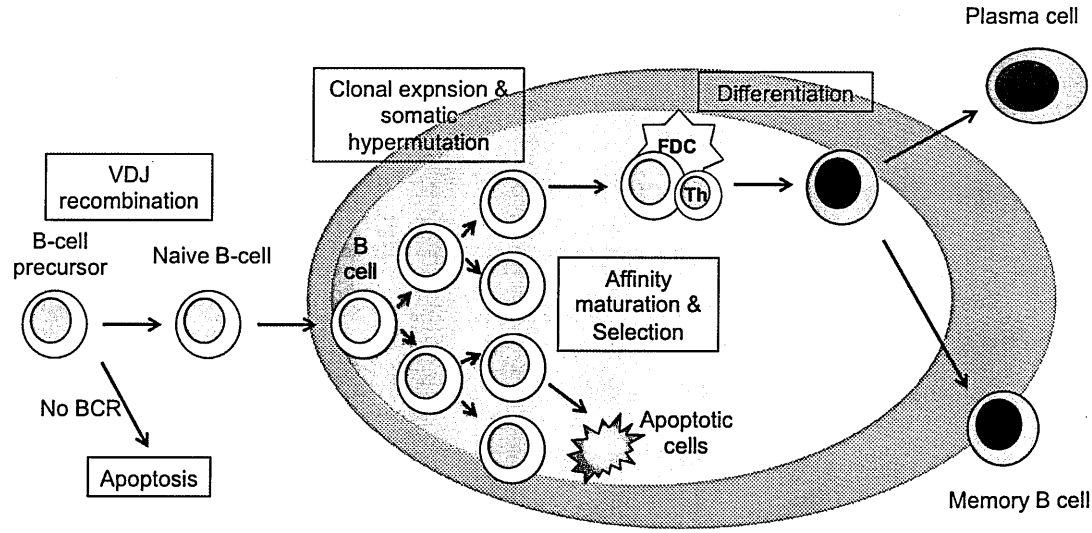


Figure 1.2 B-cell development.
Naïve antigen-activated B cells that receive "helper" signals are driven into B-cell follicles in secondary lymphoid organs, such as lymph nodes, where they establish germinal centers (GC). Proliferating GC B cells displaced the naive IgM+IgD+ B cells to the outside of the follicle, where they form a mantle zone (dotted). Within GC, a dark zone, with proliferating GC B cells (dark gray), and a light zone, containing resting GC B cells (light gray), can be distinguished (left and right sides, respectively). Proliferating GC B cells undergo somatic hypermutation in Ig V regions and, when they acquire an increased affinity for the antigen, they are positively selected through the interaction with CD4+ T cells (Th) and follicular dendritic cells (FDC) in the light zone. A fraction of these GC B cells undergo class-switch recombination, and, finally differentiate into memory B cells or plasma cells and leave the GC microenvironment.

Malignant B cells seem to be 'frozen' at a particular differentiation stage, which reflects their origin. Thus, human B-cell lymphomas are assigned to their proposed normal B-cell counterpart on the basis of the particular structure of BCR, the expression patterns of differentiation markers, and their specific tissue localization (Epstein, Küppers et al. 1999; Shaffer, Rosenwald et al. 2002; Küppers 2005) (Figure 1.3).

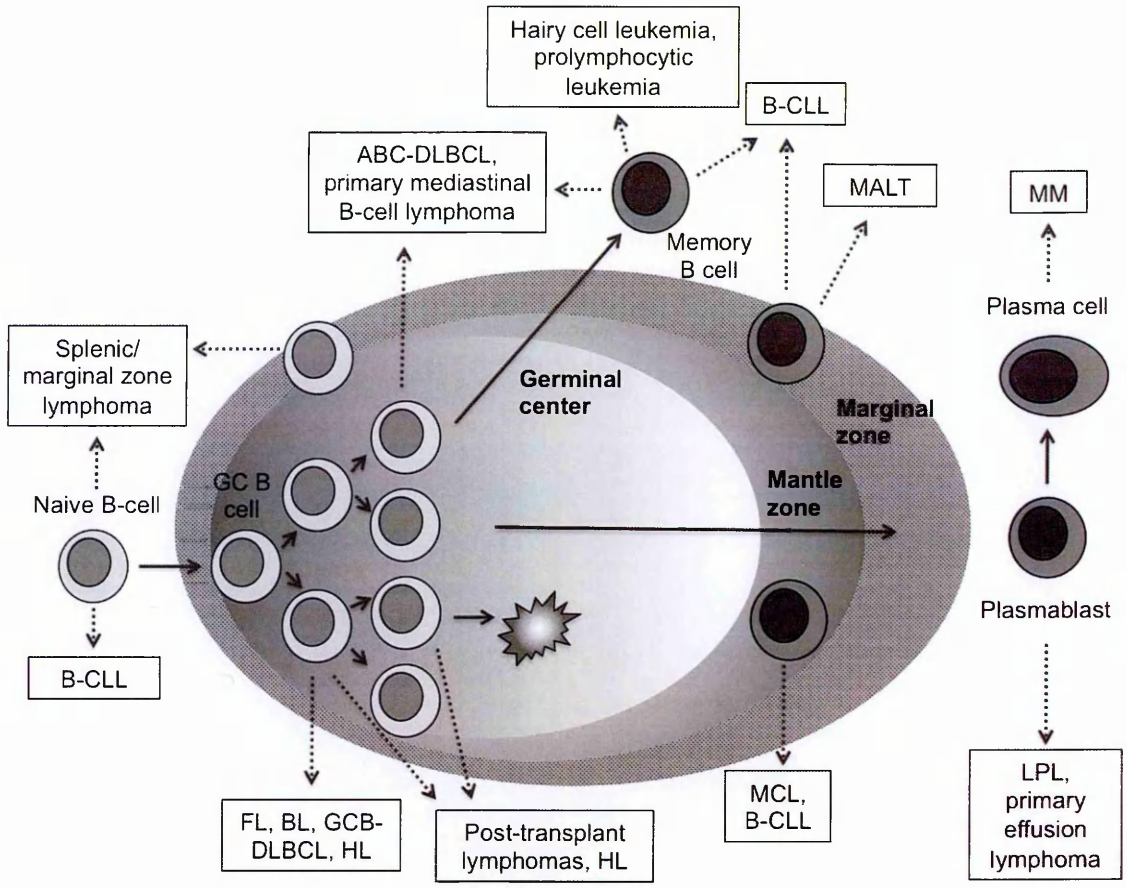


Figure 1.3 Cellular origin of human B-cell lymphomas.
Human B-cell lymphomas are assigned to their proposed normal B-cell counterpart. Most lymphomas are derived from germinal-center (GC) B cells or from B cells that have passed through the GC, indicating its role in the pathogenesis of B-cell lymphoma. FL: follicular lymphoma; BL: Burkitt's lymphoma; DLBCL: diffuse large B-cell lymphoma; GCB: germinal center B-cell like; ABC: activated B-cell like; CLL: chronic lymphocytic leukemia; MCL: mantle cell lymphoma; MZL: marginal zone lymphoma; LPL: lymphoplasmacytoid lymphoma; MM: multiple myeloma; MALT: mucose-associated lymphoid tissue lymphoma; HL: Hodgkin's lymphoma.

According to these features, the World Health Organization classification (WHO) has identified 12 subtypes of B-NHL, which are listed in Table 1.1 (Jaffe 2009). Follicular lymphoma (FL) and diffuse large B-cell lymphoma (DLBCL) are the two most prevalent NHL subtypes and together account for about 50 percent of cases. Reciprocal chromosomal translocations involving one of the Ig loci and a proto-oncogene constitute the hallmark, and thus a diagnostic marker, of many types of B-cell lymphoma (Willis and Dyer 2000; Küppers and Dalla-Favera 2001) (Table 1.1). As a consequence of the t(14;18), the BCL-2 oncogene comes under the control of the active Ig locus, causing a deregulated, constitutive expression of the oncogene.

Translocations may happen during three major steps throughout the life of a B cell: i. as mistakes occurring during Ig V(D)J gene segment recombination in early B-cell development in the bone marrow (BM) (Jäger, Böcskör et al. 2000), ii. as by-products of the somatic hypermutation process (Bross, Fukita et al. 2000), and iii. during class-switch recombination in the GC. The last two processes are typical features of B-cell development that occur exclusively in the GC (Esser and Radbruch 1990), partly explaining why B cells are more prone to undergo malignant transformation than T cells and why most B-cell lymphomas derive from GC B cells or their descendents. Besides chromosome translocations, many other transforming events have also been implicated in the pathogenesis of B-cell lymphomas, such as mutations in tumour-suppressor genes (such as

TP53 and the gene encoding IκBα), genomic amplifications (such as REL) and translocations not involving Ig loci (API2– MALT1) (Table 1.1). Finally, viruses might also be involved in the transformation of B cells. The most relevant example is EBV, which is found in nearly all endemic Burkitt's lymphomas (BL), in many post-transplant and primary effusion lymphomas (Thorley-Lawson and Gross 2004; Young and Rickinson 2004).

The survival and/or proliferation advantages provided by the constitutive expression of an oncogene and the deletion/inactivation of a tumour suppressor gene represent the driving force for the uncontrolled expansion of a B-cell clone. Carrying the same BCR on the surface, B-NHLs are distinguished by the unique antigenic determinants of BCR hypervariable regions, termed idiotype (Id), which represent a one amongst few tumour-specific antigens identified until now.

Table 1.1 B-cell lymphoma classification.

Lymphoma	Frequency among lymphoma (%)	Proposed cellular origin	Chromosome translocation (frequency)	Tumour-suppressor gene mutation (frequency)	Viruses (frequency)	Other alterations (frequency)
B-CLL	7	CD5+ small memory, naive, or marginal-zone B cells	-	ATM (30), TP53 (15)	-	Deletion on 13q14 (60)
MCL	5	CD5+ mantle-zone B cells	CCND1–IgH (95)	ATM (40)	-	Deletion on 13q14 (50-70)
FL	20	GC B cells	BCL2–IgH (90)	-	-	-
MALT	7	Marginal-zone B cells	API2-MALT1 (30), BCL10_IgH (5), MALT1-IgH (15-20), FOXP1-IgH (10)	CD95 (5-80)	Indirect role of Helicobacter Pylori in gastric MALT lymphomas	-
MZL	2	Marginal-zone or monocytoid B cells	-	-	-	-
Splenic MZL	1	Small IgD+ naive marginal-zone B cells	-	-	-	Deletion on 7q22-36 (40)
BL	2	GC B celsl	MYC-IgH or MYC-IgL (100)	TP53 (40), RB (20-80)	EBV (endemic, 95; sporadic, 30)	-
DLBCL	30-40	post-GC B cells	BCL6–various (35) BCL2–IgH (15-30) MYC-IgH or MYC-IgL (15)	CD95 (10-20), ATM (15), TP53 (25)	-	Aberrant hypermutation of multiple proto-oncogenes (50)
Primary mediastinal B-cell lymphoma	2	Thymic B cells	-	SOCS1 (40)	-	Aberrant hypermutation of multiple proto-oncogenes (40)
Post-transplant lymphoma	<1	GC B cells	-	-	EBV (90)	-
Primary effusion lymphoma	<0.5	(Post) GC B cells	-	-	HHV8 (95), EBV (70)	-
LPL; Waldenstrom's disease	1	(Post) GC B cells	PAX5–IgH (50)	-	-	-

B-CLL, B-cell chronic lymphocytic leukemia; MCL, mantle-cell lymphoma; FL, follicular lymphoma; MALT, mucosa associated lymphatic tissue lymphoma; MZL, marginal zone lymphoma; BL, Burkitt's lymphoma; DLBCL, diffuse large B-cell lymphoma; LPL, lymphoplasmacytic lymphoma; GC, germinal center.

Clinically, NHLs are classified according to the Cotswolds modification of the Ann Arbor staging system (Olweny 1990) into four stages based on anatomic sites of involvement and the presence of disease above or below the diaphragm (Figure 1.4). For each stage, lymphomas are further divided in two subsets according to the presence (A) or not (B) of systemic symptoms (night sweats, weight loss of >10% or fevers) (Figure 1.4).

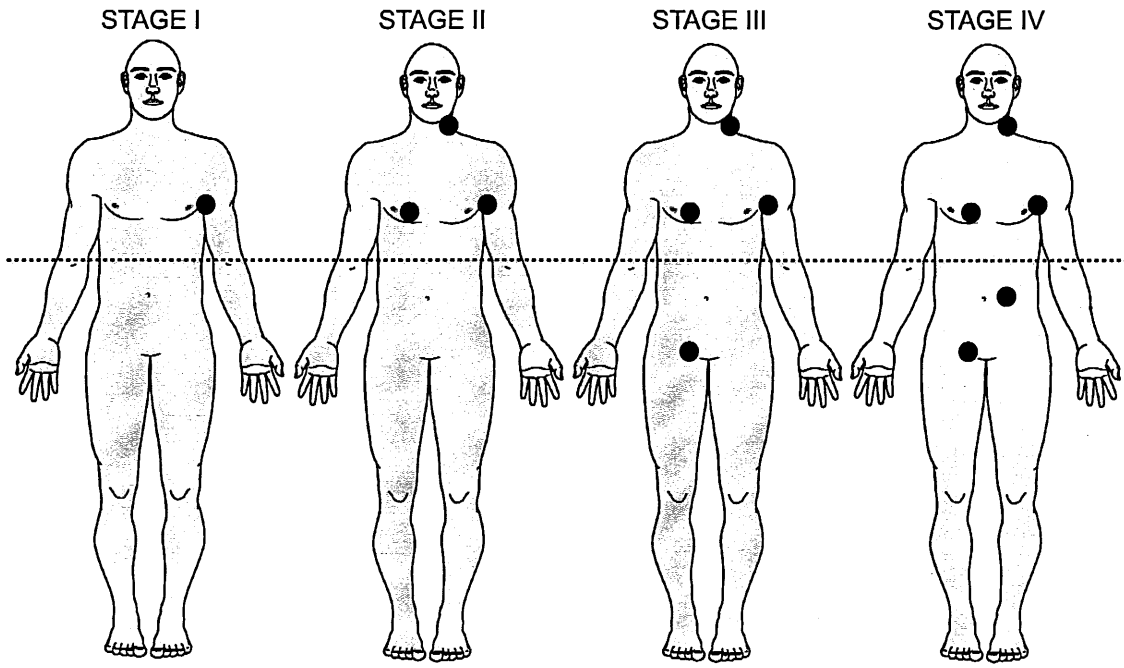


Figure 1.4 Ann Arbor staging system.
Schematic examples of stage I-IV disease is shown. Stage I: single lymph node or single extralymphatic site; stage II: two or more tumour sites in the same side with respect to the diaphragm or contiguous extralymphatic site; stage III: involvement of both site of the diaphragm or spleen, or contiguous extralymphatic sites or both; stage IV: diffuse or disseminated involvement of one or more extralymphatic sites and/or lymph nodes. Dotted line indicated the diaphragm; black filled circles represent involved tumour sites.

Commonly, NHLs are further characterized as either "aggressive" or "indolent" (http://www.nih.gov/), even though the WHO lymphoma classification (Jaffe 2009) does not include this terminology. Rapidly progressing high-grade or aggressive NHLs account about 60% of cases in the United States, with DLBCL being the most common subtype. Slow-growing indolent NHLs encompass the low-grade and some categories of intermediate-grade subtypes, with FL being the most frequent (Table 1.2). Both intermediate/aggressive and indolent diseases are diagnosed at stage III or IV in more than 50% and 80% of cases, respectively. In contrast with other types of cancer, stage IV NHL may be still highly curable, depending on the patient's specific subtype of disease. Since the subtype and stage of NHL and whether it is the indolent or aggressive form determines appropriate treatment, an accurate diagnosis is required to optimize the management of NHL patients.

Table 1.2 Frequencies of indolent lymphoma according to the WHO classification system.

	Frequency, %
Follicular Lymphoma	22
Small lymphocytic lymphoma	6
Marginal zone B-cell lymphoma, mucosa-associated lymphoid tissue type	5
Marginal zone B-cell Lymphoma, nodal type	1
Lymphoplasmacytic lymphoma	1

(Adapted from Gribben 2007)

FL represents not only the most common indolent NHL, but also the second most frequent subtype of lymphoma worldwide, accounting for approximately 20% of malignant lymphomas in adults, but 40% of all lymphomas diagnosed in the United States and in Western Europe (Armitage and Weisenburger 1998). FL is derived from GC B cells and maintains the gene expression profile of this stage of differentiation (Dave, Wright et al. 2004). Morphologically, the disease is composed of a mixture of centrocytes and centroblasts and is graded from 1 to 3, depending on the proportion of large cells per high-power field. Grades 1 and 2 are indolent disease. The rare subtype grade 3b is more aggressive and should be discriminated from lower-grade cases.

FL cells express a surface Ig (more frequently IgM+/−IgD > IgG > IgA), B-cell-associated antigens (CD19, CD20, CD22, CD79a and CD79b), and 60% express CD10. They are CD5−, CD23−/+, CD43−, and CD11c−. CD5 and CD43 negativity is useful in differentiating FL from chronic lymphocytic leukemia (CLL) and MCL, whilst CD10 reactivity is useful in distinguishing MZL (Vitolo, Ferreri et al. 2008). The hallmark of FL is the chromosomal translocation t(14;18), present in 70–95% of the cases, which results in the constitutive expression of the antiapoptotic protein BCL2, and thus allows to distinguish reactive from neoplastic follicles (Ngan, Chen-Levy et al. 1988; Pezzella, Tse et al. 1990).

FL can involve every anatomic district, but the most common presentation is multiple lymphoadenopathy. Advanced disease is found in 80–85%, 70–75% and 65–70% of grade 1, 2 and 3 cases respectively. As other indolent lymphomas, FL is characterized by extreme and often unpredictable clinical variability, with a continuous pattern of relapse that sometimes leads to a rapid unexpected clinical worsening, and remains incurable with the available therapies (Hiddemann, Buske et al. 2005). As an example, 15-60% FL undergo transformation in a largely unpredictable way into the more aggressive histologic malignancy DLBCL, following molecular mechanisms that are not entirely known (Horning and Rosenberg 1984; Lossos 2005; Rosenberg 2008). Transformed lymphomas are usually quite aggressive and poorly responsive to chemotherapy (O'Brien, Easterbrook et al. 1991), with a median survival from transformation of about 18 months (Rohatiner and Lister 2005; Montoto, Davies et al. 2007). Thus, the accurate biomolecular characterization of tumour features associated with progression or response after therapy is essential to identify predictive and prognostic biomarkers and molecular targets for new drug development, thereby enabling the improvement of the clinical management for indolent NHL patients.

Multiple lines of evidence also point to a crucial dependence of indolent lymphomas on infiltrating immune cells for its maintenance and progression (Herreros, Sanchez-Aguilera et al. 2008). An important example is the demonstration that removing bacteria or viruses, such as Helicobacter pylori and hepatitis C virus and EBV, from the lymphoma

microenvironment may efficiently arrest tumour growth (Wotherspoon, Doglioni et al. 1993; Hermine, Lefrere et al. 2002; Sagaert, De Wolf-Peeters et al. 2007). Tumour-infiltrating immune cells, including T lymphocytes, macrophages and dendritic cells (DCs), can provide contact-dependent or independent signals for lymphoma cell survival (de Jong 2005; Dave 2008). Proliferation of B-CLL cells is largely restricted to proliferation centres in lymph nodes and BM, where neoplastic cells are in intimate contact with nurse-like cells (NLCs), CD4+ T cells and DCs that prevent apoptosis and/or trigger survival of tumour B cells (Figure 1.5A) (Schmid and Isaacson 1994; Ghia, Circosta et al. 2005; Nishio, Endo et al. 2005). Several findings indicate the participation of immune cells also in the biology and pathogenesis of FL (Alvaro, Lejeune et al. 2006a; Glas, Knoops et al. 2007). FL cell growth depends on stromal cells and, in particular, upon follicular DC CD40 stimulation that can prevent caspase activation and the spontaneous apoptosis of indolent lymphoma cells (Figure 1.5B) (Eray, Postila et al. 2003; Goval, Thielen et al. 2008). Noteworthy, the gene and immunohistochemical signature of non-tumour cells in the neoplastic tissue currently represents the best predictor for FL patient survival (Figure 1.5B) (Dave, Wright et al. 2004; Farinha, Masoudi et al. 2005; Lee, Clear et al. 2006; Glas, Knoops et al. 2007).

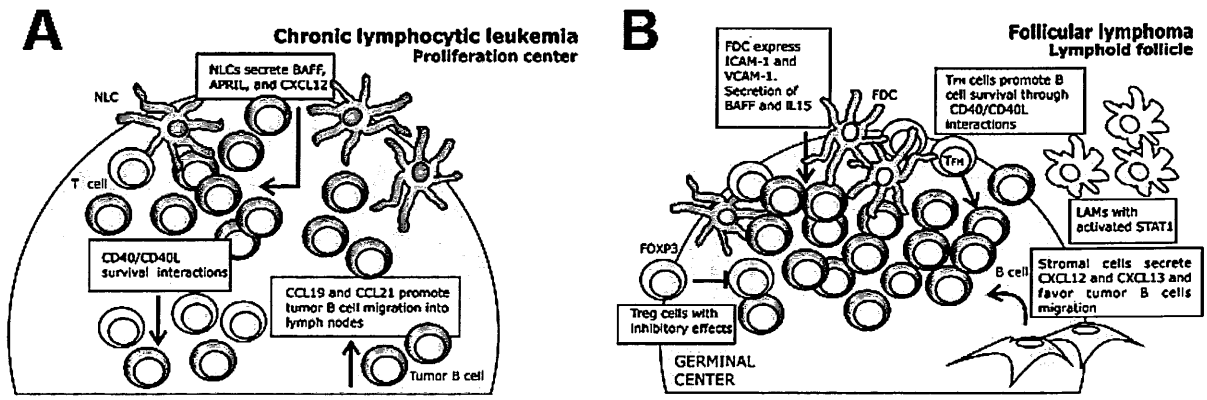


Figure 1.5 Lymphoma microenvironment.
(A) Chronic lymphocytic leukaemia cells proliferate in transformation centres responding to environment signals. NLCs, nurse-like cells. (B) Survival of tumour cells in FL depends on their interaction with multiple subpopulations of specialized T cells, macrophages and dendritic cells. GC, germinal centre; Treg, regulatory T cells; TFH, follicular B-helper T cells; LAMs, lymphoma associated macrophages. (Adapted from Herreros 2008)

The dysfunctional immune profiles in FL microenvironment can also reprogram other immune cells such as T regulatory cells (Tregs) and lymphoma-associated macrophages (LAM). FL cells can attract or locally convert FOXP3+ Tregs that in turn suppress anti-tumour immunity directly by impairing T cell activation (Yang, Novak et al. 2006; Yang, Novak et al. 2007; Ai, Hou et al. 2009) and through the induction of M2 polarization of LAM, amplifying the tumour immune evasion (Farinha, Masoudi et al. 2005; Alvaro, Lejeune et al. 2006b; Tiemessen, Jagger et al. 2007). However, published data on the prognostic significance of the presence of Tregs within FL microenvironment are highly conflicting (Gribben 2010) (Table 1.3).

Table 1.3 Immune microenvironment in FL.

Study	FOXP3 *few*	FOXP3 *many*	FOXP3 *Intra-follicular*	CD68 *many*	CD4 *few*	CD4 *many*	CD4 *Inter-follicular*	CD8 *few*	CD21/23 *disrupted*
Farinha P, Blood 2010	good	poor	poor	poor	-	ND	ND	-	ND
Carreras J, Blood 2006	poor	good	good	-	-	ND	ND	ND	ND
Lee AM, J Clin Oncol 2006	poor	good	good	-	poor	good	good	-	ND
Alvaro T, J Clin Oncol 2006	poor	good	good	good	poor	good	-	poor	ND
Glas AM, J Clin Oncol 2007	-	-	-	-	-	-	good	-	poor

(Adapted from Gribben 2010)

The highly heterogeneous treatments used in these studies may partly explain the observed discrepancies since they have shown to differentially impact the immune cells within FL microenvironment (de Jong, Koster et al. 2009). A very recent study conducted in a series of uniformly treated patients has evidenced that, more than Treg amount, their architectural distribution within FL lymphoma microenvironment plays a key role in predicting not only survival but also the risk of transformation (Farinha, Al-Tourah et al. 2010). Therefore, the concomitant study of the intrinsic tumour features, the functional composition of the non-malignant microenvironment, and constitutive patient-related properties may be very useful to improve the management of indolent NHL patients and develop alternative therapies aimed at interfering with the pro-survival interaction between tumour and inflammatory cells.

1.1.2 Standard therapeutic approaches

Due to the indolent course of low-grade lymphoma, a major clinical question is how to identify the patients that may benefit from early therapy (Gribben 2007). The institution of therapy for indolent NHLs currently depends on patients' prognostic/risk factors, measured according to the FL International Prognostic Index (FLIPI) score that takes into account patients' age (>60 years), stage of the disease, number and size (>6 cm) of involved nodal sites, BM involvement, haemoglobin (<120 g/dl), LDH and β2-microglobulin levels (Solal-Celigny, Roy et al. 2004; Buske, Hoster et al. 2006; Federico, Bellei et al. 2009). Using these parameters, 3 risk groups have been identified, with 91, 69, and 51% progression free survival (PFS) and 99, 96, and 84% survival rate at 3 years for patients at low, intermediate, and high risk, respectively. Although FLIPI score helps to risk-stratify patients, there is a need for robust biomarkers of disease outcome.

The identification of a gene expression prognostic signature related to tumour infiltrating immune cells can provide additional important information to predict survival. However, at present these factors cannot yet be considered, since they need to be further corroborated before entering the clinical practice (Dave, Wright et al. 2004; Farinha, Masoudi et al. 2005; Carreras, Lopez-Guillermo et al. 2006; Lee, Clear et al. 2006). Expectant management ("watch and wait" approach) is usually considered for asymptomatic patients with low-bulk disease until clear indications for treatment initiation (Figure 1.6) (Vitolo, Ferreri et al. 2008). Indeed, no survival advantage has been demonstrated when patients in these condition have been immediately treated (Hoppe, Kushlan et al. 1981).Once treatment is indicated, many therapeutic approaches are available (Table 1.4).

Table 1.4 Treatment strategies for indolent lymphomas.

Advanced stage disease
Watchful waiting
Alkylating agents
Purine analogs
Combination chemotherapy
Monoclonal antibodies • Unconjugated • Conjugated: radioimmunoconjugates, immunotoxins
Chemotherapy + monoclonal antibodies (chemoimmunotherapy)
High-dose chemotherapy + autologous/allogeneic stem cell transplantation
Reduced intensity conditioning allogeneic stem cell transplantation
Palliative radiotherapy
Localized disease
Radiotherapy
Watchful waiting

(Adapted from Gribben 2007)

Combination chemotherapy regimens including alkylating agents, such as chlorambucil and cyclophophamide (CVP: cyclophosphamide, vincristine, and prednisone;

or CHOP: cyclophosphamide, doxorubicin / hydroxydoxorubicin, vincristine and prednisone), have been the standard of therapy for indolent lymphomas for decades (Lister, Cullen et al. 1978; Dana, Dahlberg et al. 1993; Peterson, Petroni et al. 2003). Purine analogues (i.e. Fludarabine) have been also extensively studied, and their combination with cyclophosphamide (Hochster, Oken et al. 2000; Eichhorst, Busch et al. 2006) and mitoxantrone (Velasquez, Lew et al. 2003) have provided higher response rates compared to either agents alone. However, since the introduction of monoclonal antibodies (mAbs) for the treatment of lymphoma, the standard of choice as first-line therapy has been their combination with chemotherapy, namely chemoimmunotherapy (Keating, O'Brien et al. 2005).

The most widely used mAb is rituximab, a chimeric unconjugated Ab against the CD20 antigen licensed by the Food and Drug Administration (FDA) (Food and Drug Administration. Draft labeling text for Rituxan (rituximab). http://www.fda.gov/cder/foi/label/2006/103705s5230-s5231lbl.pdf. Accessed February 2, 2007) and the European Agency for the Evaluation of Medicinal Products (European Medicine Agency. European Public Assessment Report for Mabthera. http://www.emea.eu.int/humandocs/Humans/EPAR/mabthera/ mabthera.htm. Accessed February 2, 2007) to treat DLBCL as well as relapsed or refractory, low-grade CD20+ B-NHLs as a single agent; non-progressing (including stable disease), low-grade, CD20+ B-NHLs, as a single agent, after first-line CVP chemotherapy; previously untreated indolent, CD20+ B-NHLs in combination with CVP chemotherapy (Leget and Czuczman 1998). Multiple randomized clinical trials have demonstrated a significant survival benefit with the addition of rituximab to first-line chemotherapy in patients with FL and DLBCL (Press, Leonard et al. 2001; Vose, Link et al. 2001; Hiddemann, Kneba et al. 2005; Marcus, Imrie et al. 2005; Herold, Haas et al. 2007; Schulz, Bohlius et al. 2007; Tan and Horning 2008). Rituximab has also been evaluated as first-line monotherapy treatment of low-grade NHLs, showing encouraging results (Hainsworth 2000; Hainsworth, Burris et al. 2000; Colombat, Salles et al. 2001; Solal-Celigny 2001).

More aggressive front-line therapies are usually considered for patients with a rapidly progressing disease, which represents an indicator of poor prognosis (Brice, Bastion et al. 1997).

According to performance status, previous therapy, response, and duration of response, it is possible to define treatment at relapse (palliative versus potentially curative). Therefore, relapsed indolent NHLs are again managed expectantly or are treated with rituximab as single agent (McLaughlin, Grillo-Lopez et al. 1998; McLaughlin, Hagemeister et al. 1999) or in combination with chemotherapy; alternatively, relapsed patients can enter clinical trials for the study of new biotherapies (Figure 1.6). Autologous haematopoietic stem cell transplantation (HSCT) is applicable in only a minority of patients, due to extensive prior therapy and frequent marrow involvement. However, encouraging results have been obtained when autologous HSCT has been instituted for young patients with chemo-sensitive disease. (Freedman, Ritz et al. 1991; Freedman, Neuberg et al. 1999; Apostolidis, Gupta et al. 2000; Rohatiner, Nadler et al. 2007) (Figure 1.6). Even though allogeneic HSCT is associated with a higher transplant related mortality, it is increasingly used as salvage therapy for relapsed or refractory indolent lymphomas, due to the lower risk of relapse, and the long-term progression free survival (PFS) observed after allogeneic compared to autologous HSCT (Toze, Barnett et al. 2004) (Figure 1.6). Donor lymphocyte infusion (DLI) has demonstrated a significant therapeutic activity in case of relapse after allogeneic HSCT, thus pointing to the efficacy of a graft-versus-lymphoma (GVL) effect in indolent lymphomas (Morris, Thomson et al. 2004; Gribben, Zahrieh et al. 2005). To better exploit the GVL activity of alloreactive donor T cells, nonmyeloablative or reduced-intensity conditioning regimens prior to allogeneic HSCT have been recently introduced in the clinical practice. This strategy has decreased the acute mortality from conditioning

toxicity and can be used for the treatment of elderly patients and patients with significant co-morbidities (Khouri, McLaughlin et al. 2008; Rezvani, Storer et al. 2008). However, the risk of both acute and chronic graft versus host disease (GVHD) or late non-relapse deaths due to immunosuppression from GVHD treatment continue to exist, and the optimal scheduling of such a life-threatening but potentially curative therapy remains challenging. In sharp contrast, when newly diagnosed indolent NHL is limited (stage I or II), immediate involved-field radiation therapy (IFRT) is usually recommended since it has the potential to eradicate the disease (Vaughan Hudson, Vaughan Hudson et al. 1994; Wilder, Jones et al. 2001) (Figure 1.6).

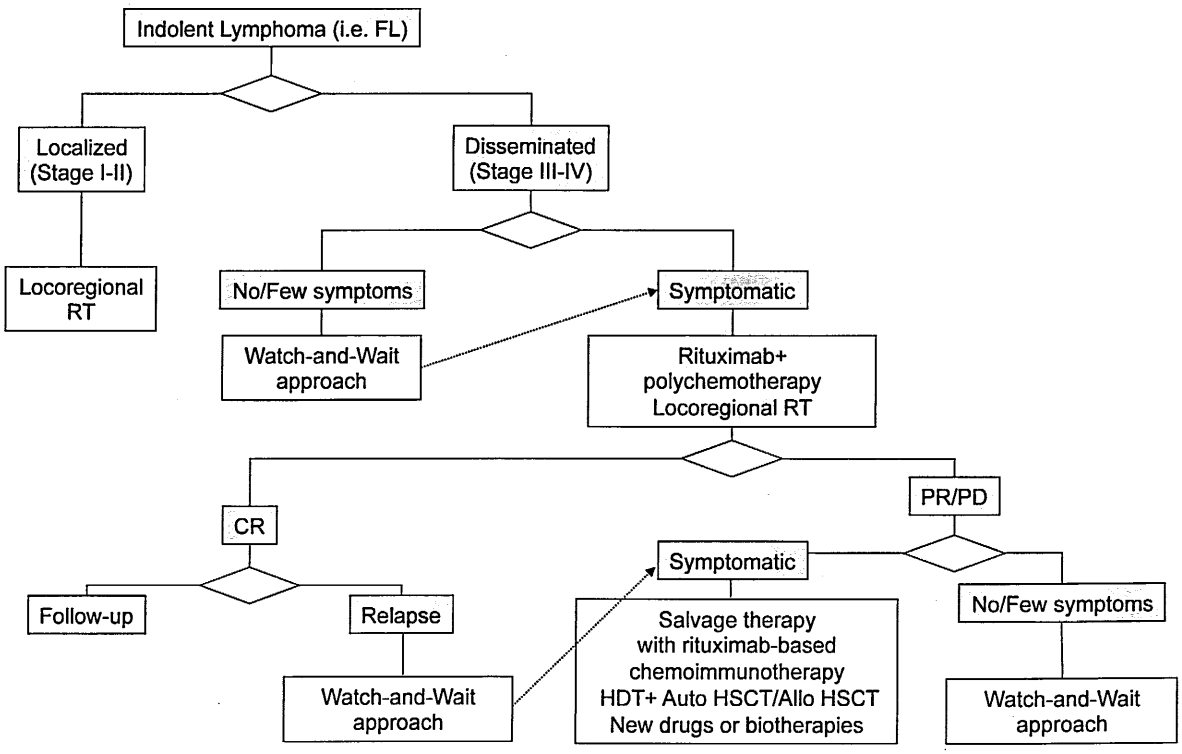


Figure 1.6 Therapeutic algorithm for the treatment of indolent NHLs.
RT: radiotherapy; HDT: high-dose therapy; Auto HSCT: autologous hematopoietic stem cell transplantation; Allo HSCT: allogeneic hematopoietic stem cell transplantation; CR: complete remission; PR: partial remission; PD: progressive disease

More extensive nodal radiation as well as the addition of chemotherapy to radiation therapy does not seem to improve overall survival (Matasar and Zelenetz 2008). An excellent therapeutic alternative for any lines of treatment of indolent NHLs is the enrolment of the patients in randomized clinical trials due to the slow-progressing nature of the disease and the possibility to keep it in check.

Several lines of evidence have pointed to a high susceptibility of lymphoma to immunotherapeutic approaches such as passive immunotherapy with different mAbs, radioimmunotherapy, vaccine-based treatments, HSCT and adoptive cellular therapy. FL B-NHL has long been regarded as particularly immune responsive based on reports of spontaneous regressions (Horning and Rosenberg 1984) and high response rates to mAbs (McLaughlin, Grillo-Lopez et al. 1998; Forstpointner, Unterhalt et al. 2006). Accordingly, over the past 30 years only the introduction of chemoimmunotherapy with rituximab has recently demonstrated to improve the overall survival (OS) of FL patients, indicating that the natural history of this disease is potentially changing (Fisher, LeBlanc et al. 2005; Friedberg 2008). However, resistance to rituximab remains a problem (Hainsworth, Litchy et al. 2005) and regimens that improve remission duration are needed. Novel treatments,

without the use of chemotherapy, such as immunotherapeutic strategies, may provide opportunities for the cure of relapsed/refractory indolent NHLs.

1.2 Anticancer immunotherapy

The term immunotherapy describes multiple approaches aiming at conferring either passive or active immunity. Passive immunity supplies the immune response through the infusion of Abs or cytotoxic T cells, with the major limitation that it is short-lived. By contrast, active immunity is though to stimulate an endogenous immune response, where the immune system is primed to recognize the tumour as foreign, and, thus, represents the ideal form of immunotherapy. It is now generally agreed that the future of cancer therapy lies in the combination of therapies with different mechanisms of action. Immunotherapy is increasingly used in combination with traditional treatments in comprehensive immunotherapeutic programmes with the aim to attack cancer cells on multiple sides and promote the onset of specific immunity to avoid recurrence (Andersen, Junker et al. 2010).

The rationale to use immunotherapy for the treatment of cancer relies on the concept of cancer immunosourveillance (Sahin, Türeci et al. 1995; Stockert, Jager et al. 1998), in which the host immune system acts as an extrinsic tumour suppressor. In cancer patients, however, this mechanism is gradually subverted through the process of immunoediting by which tumour cells reduce their immunogenicity. Malignant cells that can escape immune recognition are thus positively selected and promote the outgrowth of the tumour (Figure 1.7) (Dunn, Old et al. 2004). There may be multiple evasive mechanisms adopted by the malignant cells to impede proper immune activation. Examples include the down-regulation of tumour antigens and major histocompatibility complex (MHC) molecules, the establishment of a tolerogenic environment by secreting immunosuppressive molecules that affect antigen presenting cells (APCs) maturation, differentiation, and activity and by enhancing Treg activity (Zou 2005).

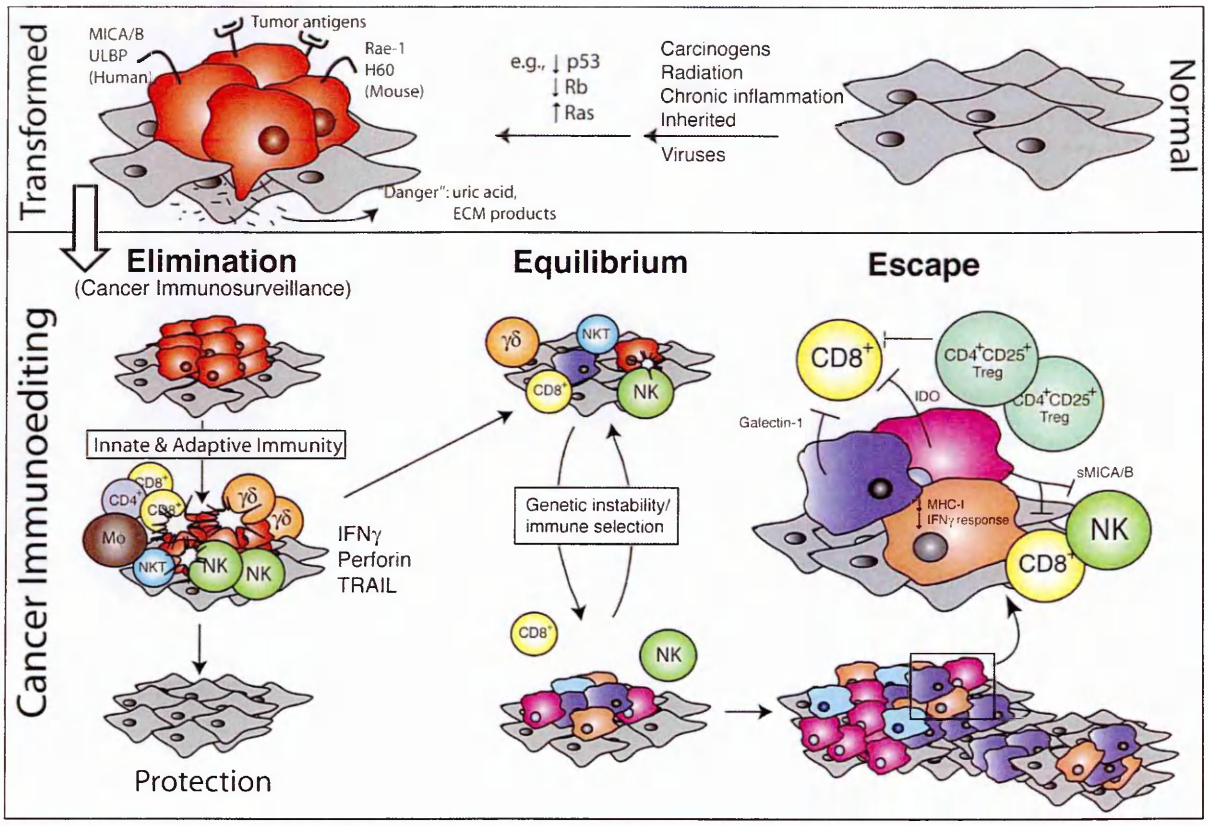


Figure 1.7 Dynamic model of tumour immunosourveillance.

Following cellular transformation mechanisms that lead to the development of a neoplastic clone, its ultimate fate depends on immunosuorveillance: it may be eliminated (Elimination phase), maintained in a dormant/equilibrium state (Equilibrium phase) or escape the host protective actions of immunity by either becoming non-immunogenic or through the elaboration of immunosuppressive molecules and cells (Escape phase). (Adapted from Dunn 2004).

Immunotherapy has thus the aim to re-establish or supply for the host immune functions that allow the patients to recognize tumours as dangerous, mount a full-fledged attack, and eradicate the cancerous cells with less toxic effects than using conventional radiotherapy, chemotherapy and surgery.

Thus far, melanoma has been the target for the majority of studies for the potential efficacy of immunotherapy due to its intrinsic high immunogenicity and sensitivity to a specific immune attack. Indolent lymphomas, however, remain one of the most suitable diseases that may benefit from this kind of intervention for two main reasons. First, more than other malignancies, they grow thanks to the alteration of the balance between immunity and tolerance in the tumour microenvironment; second, their indolent course leaves optimal therapeutic windows for the study of potentially useful immunotherapeutic interventions with minimal side effects. In the past 15 years a number of immunologically based treatment options have been developed for patients with indolent lymphoma. To date, targeting CD20 on the surface of malignant B cells with mAbs, in particular rituximab, has been the most clinically effective strategy (Coiffier 2007). Patient-specific vaccines targeting the clonally derived Ig-Id protein have also been largely exploited to induce an endogenous adaptive anti-lymphoma response able to clear cancer cells and establish an anti-tumour immunological memory (Park and Neelapu 2008). Lastly, adoptive transfer of anti-tumour cytotoxic T lymphocytes (CTLs) has been used for indolent lymphoma patients on the basis of the high sensitivity of FL to GVL following allogeneic SCT and the clinical benefit associated to donor lymphocytes infusion (DLI) even in highly chemotherapy-refractory lymphomas (Khouri, McLaughlin et al. 2008; Rezvani, Storer et al. 2008). At present the major obstacle to the biological and clinical efficacy of anti-cancer immunotherapy remains immune tolerance that protects neoplastic cells from immune system attack. The molecular obstacles to the successful reversal of tolerance and induction of effective immunity are becoming clear and novel immunotherapeutic strategies should therefore consider multiple elements to overcome them, whilst providing a durable anti-tumour immune response that could be ideally maintained throughout patient's lifespan.

1.2.1 Passive Immunotherapy

Passive immunotherapy does not stimulate the patient's immune system to "actively" respond to a disease in the way a vaccine does. It rather consists of the administration of mAbs produced by hybridoma or recombinant protein technology to react against specific antigens on cancer cells, such as a receptor, a specific enzyme or a protein. Binding of the Ab may then activate effector cells in the host immune system (Hjelm, Carlsson et al. 2006).

MAbs are thus a form of targeted therapy that, following reaction with the specific antigen, can exert protective functions in three principal ways. They can activate the complement system that, forming a membrane attack complex on the target cells, causes their lysis and death (complement dependent cytotoxicity (CDC) (Reff, Carner et al. 1994), or favours their opsonization and phagocytosis by neutrophils and macrophages. In addition phagocytes can be directly recruited to the Ab Fc via the specific receptor and eat the bound cell (Ab-dependent cell mediated cytotoxicity (ADCC)) (Clynes, Towers et al. 2000). ADCC has the advantage of catalyzing T cell activation, as the digested foreign cell proteins are presented on the MHC molecules of APCs as peptides. Finally, depending on the targeted antigens, Abs have also been shown to kill cancer cells by blocking growth mechanisms or directly inducing apoptosis as a conventional targeted therapy does.

With the advent of mAb technology (Kohler and Milstein 1975; Stashenko, Nadler et al. 1980) more than ten cell-surface molecules have been identified on B cells, with different expression levels at various stages of their development. However, most of these antigens are found also on the malignant B-cell counterpart, and have been thus exploited as molecular targets for mAb therapy (Figure 1.8).

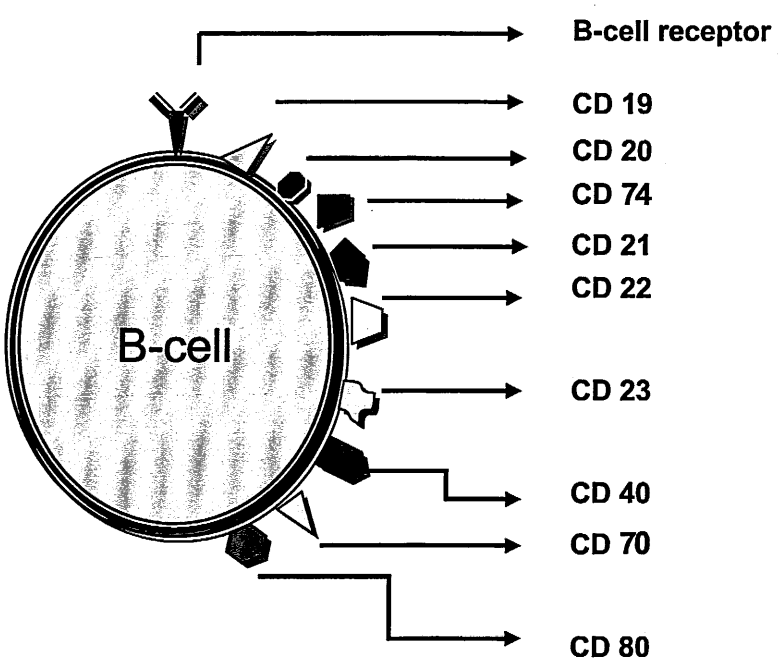


Figure 1.8 B-cell antigen targets.
B-cell surface-associated antigens that have been exploited as immunotherapeutic targets for the treatment of B-cell malignancies. (Adapted from Tay 2010)

To date, CD20 has been the most commonly targeted antigen by mAb therapy for B-cell malignancies because of its advantageous characteristics of being expressed at high levels on the surface of most malignant B cells, where it is tightly bound with little modulation and no secretion or rapidly shedding in circulation. Furthermore CD20

expression is absent on the early B-cell precursors allowing repopulation of the B-cell compartment after treatment (Cragg, Walshe et al. 2005). In addition, CD20 seems to have an important role in the physiology of B-cell activation and cell-cycle regulation (Jazirehi and Bonavida 2005). Thus, apart from its binding to normal B cells, CD20 represents an ideal target for B cell lymphoma.

The chimeric human–mouse IgG1 kappa anti-CD20 mAb rituximab was the first mAb to be approved by the Food and Drug Administration in 1997 for treatment of relapsed or refractory CD20-positive indolent B-NHLs (Food and Drug Administration, http://www.fda.gov/cder/ foi/label/2006/103705s5230-s5231lbl.pdf) (Leget and Czuczman 1998). On binding to CD20, rituximab induces ADCC, CDC, apoptosis of neoplastic B cells by promoting calcium influx and caspase activation (Golay, Zaffaroni et al. 2000; Pedersen, Buhl et al. 2002) and sensitizes malignant B cells to chemotherapy (Alas, Emmanouilides et al. 2001). Rituximab has been the most valuable addition to the treatment for B-NHLs since 20 years and is now considered the cornerstone of therapy in both indolent and aggressive B-cell lymphomas. Nevertheless, about 30% and 50% of FL patients do not respond to rituximab in first-line or more advanced treatment settings respectively (McLaughlin, Grillo-Lopez et al. 1998), and approximately 60% of initial responders no longer benefit from retreatment (Davis, Grillo-Lopez et al. 2000). The success of rituximab, but also its recognized limitations, has stimulated investigational efforts to develop newer-generation anti-CD20 mAbs as well as mAbs targeting different surface antigens expressed on malignant B cells (Table 1.5 and 1.6, Figure 1.9). Several approaches are under evaluation, including humanization of the molecules to decrease infusion reactions and immunogenicity, while improving side-effect profile, and enhancement of binding affinity. Modification of the Fc portion is also being performed to optimize effector functions, particularly ADCC.

Table 1.5 Anti-CD20 mAb in clinical development.

mAb	Format	Indication	ADCC	CDC	Direct effects	Ag Binding	Dev. Phase
Rituximab	Chimeric IgG1	NHL/RA	++	++	+	+	Approved 1997
^{90}Y-Ibritumomab tiuxetan	Murine (^{90}Y)	NHL	+/-		+++++		Approved 2002
^{131}I-Tositumomab	Murine (^{131}I)	NHL		+/-	+++++		Approved 2003
Ofatumomab (HuMax-CD20)	Human IgG1	NHL/RA	++	++++	+	++	Phase III trials
Ocrelizumab	Humanized IgG1	NHL/RA	+++	+/-	+		Phase III trials
TRU-015	SMIP	RA	++++	+/-			Phase I/II trials
Veltuzumab	Humanized IgG1	NHL/ITP	++	++	+	=	Phase I/II trials
AME-133v	Humanized IgG1	Relapsed NHL	++++	++	++	++	Phase I/II trials
PRO13192 (rhumAb v114)	Humanized IgG1	CLL/NHL	++++	++++	=	++	Phase I/II trials
GA101	Humanized IgG1	CLL/NHL	+++++	-	++++	=	Phase I/II trials

ITP: Immune thrombocytopenic purpura; SMIP: Small Modular Immunopharmaceuticals; RA: Rheumatoid Arthritis

Second-generation anti-CD20 mAb include ofatumomab, veltuzumab, ocrelizumab that are humanized or fully human IgG1, with an unmodified Fc region (Table 1.5, 1.6 and Figure 1.9).

Given the finding that the V158F polymorfism on Fc receptor FcγRIII can impair rituximab-mediated ADCC (Cartron, Dacheux et al. 2002; Weng and Levy 2003), third-generation mAbs have been developed with an Fc region designed to bind with equal affinity mutated FcγRIII on NK cells, thus enhancing their effector functions and their therapeutic activity. Three such engineered anti-CD20 Abs - AME-133v (Bowles, Wang et al. 2006), PRO131921 (rhuMAb v114) (A Trial of the Safety of Escalating Doses of PRO131921 in Patients With Relapsed or Refractory Indolent Non-Hodgkin's Lymphoma. 2009. (Accessed 20 May 2009, at http://clinicaltrials.gov/ct2/show/NCT00452127?term=PRO131921&rank=2.), and GA-101 (Salles 2009) - are currently in early phases of clinical development. All of them have demonstrated an increased capability to mediate ADCC as compared with rituximab, and GA-101 have also shown to better induce direct apoptosis in malignant B cells (Table 1.5).

Novel anti-CD20 mAbs have demonstrated the same favourable toxicity profiles as rituximab. In general, pharmacodynamic parameters do not correlate with response, and

no clear dose-effect relationships have been detected. They are associated with significant efficacy, although response rates in rituximab-refractory patients are modest.

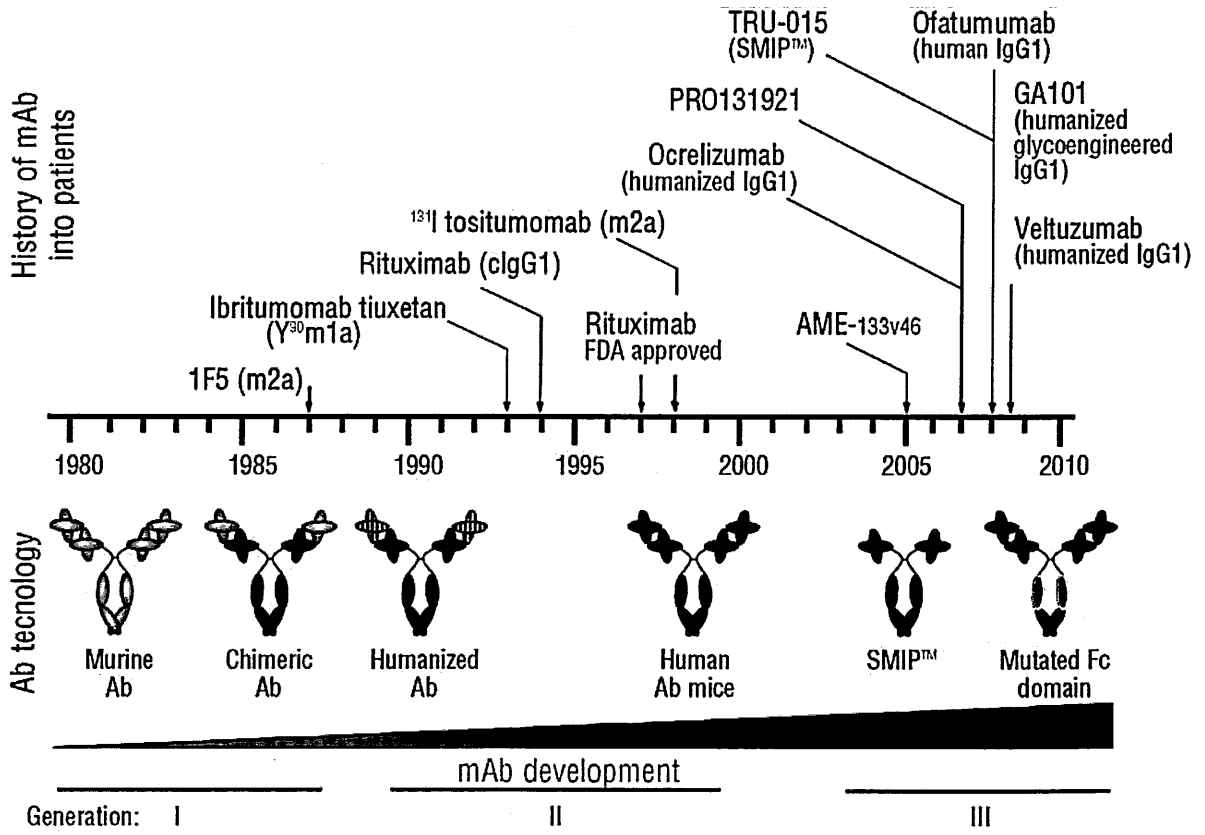


Figure 1.9 History of anti-CD20 mAb in clinical translation.
Chronological introduction of anti-CD20 mAb in clinical trials, as well as the corresponding technologic progress for their development (generation I reagents: murine or human/mouse chimeric mAbs; generation II reagents: humanized or fully human mAbs; generation III reagents: Abs with modified structures, such as mutation or a-fucosylation of the Fc domain for enhanced FcR binding profiles) (Adapted from Lim 2010).

MAbs therapies for B-NHLs targeting neoplastic B-cell antigens distinct from CD20 are developing exponentially in number with the aim of identifying more active and specific treatments. CD22, CD23, CD40, CD80, CD70, CD74 are amongst the most widely exploited biotargets for novel anti-lymphoma passive immunotherapy (Figure 1.8). However, being involved in the normal B-cell functions, these antigens are not tumour specific. Nevertheless, the humanized anti-CD22 monoclonal IgG epratuzumab (Leonard, Coleman et al. 2003), the primate-human chimeric anti-CD23 and anti-CD80 monoclonal IgG1 lumiliximab (IDEC-152) (Pathan, Chu et al. 2008) and galiximab (Czuczman, Thall et al. 2005) have shown promising clinical results and a good safety profile in phase I/II trials for relapsed/refractory indolent NHLs (Table 1.6). Moreover, the combinations of epratuzumab or galiximab with rituximab demonstrated significant clinical activity with limited toxicity (Leonard, Coleman et al. 2005) (Czuczman 2008), with the latter showing even better outcomes than either agent alone (Friedberg 2008). Epratuzumab or lumiliximab with chemoimmunotherapy or standard chemotherapy proved feasible and safe approaches for NHL patients, which compared also favourably with historical controls receiving the same treatment without these novel agents (Micallef 2009; Byrd 2006). Such encouraging results have prompted the initiation of randomized phase III trials comparing rituximab with or without galiximab and (fludarabine, cyclophosphamide, rituximab) FCR regimen with and without lumiliximab.

Due to the crucial role of CD40 engagement on the surface of lymphoma B cells for their proliferation and survival (Grdisa 2003; Kater, Evers et al. 2004), CD40 has been largely studied as a potential biotarget for these diseases. HCD122 (formerly CHIR-12.12) and dacetuzumab (SGN-40) are respectively a fully-human and a humanized anti-CD40 monoclonal IgG1 that, blocking CD40/CD40L-mediated signalling, activate proapoptotic signal transduction pathways and provide ADCC, resulting in clearance of malignant B cells (Law, Gordon et al. 2005; Luqman, Klabunde et al. 2008). Clinical evaluation of the tolerability of HCD122 is underway in phase I trials for NHLs or Hodgkin's lymphomas, multiple myeloma, and relapsed CLL. Dacetuzumab has already reached phase II clinical trials that indicate some effects as monotherapy in patients with heavily pretreated DLBCL (Advani 2008) (Table 1.6). Ongoing studies are exploring its activity in combination with conventional therapies in multiple myeloma, DLBCL, and low-grade NHLs.

MAbs targeting CD70 and CD74 are the last ones entering preclinical and initial clinical development for the treatment of B-cell malignancies (Kaufman 2009). CD70 has the advantage of being expressed prevalently on neoplastic B cells, and rarely on normal B or T lymphocytes (Hintzen, Lens et al. 1994). CD70 targeting on lymphoma cells with the specific fully-human MDX-1411 mAb has resulted in potent ADCC induction (Israel, Gulley et al. 2005). CD74 is a type-II transmembrane chaperone molecule, expressed on normal and malignant B cells, monocytes and histiocytes. It mediates several pro-survival signals and favours tumour immune escape by impairing the binding of antigenic peptides to human leukocyte antigen system (HLA)-DR (Roche and Cresswell 1990; Starlets, Gore et al. 2006). The anti-CD74 mAb Milatuzumab (hLL1, IMMU-115) has shown to cause very modest ADCC or CDC, but in the presence of an appropriate cross-linking agent, it can inhibit cell proliferation and promote apoptosis *in vitro*, resulting in significant prolongation of survival in lymphoma mouse models (Stein, Qu et al. 2004; Stein, Mattes et al. 2007).

All these results have been seen with great enthusiasm, even though, as yet, none have demonstrated significant efficacy over that seen with rituximab. Retrospective analysis of failures and successes of mAb therapy has resulted in the reemphasis of three major caveats of this approach: (i) identity of the target antigens, (ii) the limited understanding of the mechanisms of action of the mAbs and (iii) resistance induction (Maloney 2007). Tumour specificity, essential biologic activity, the absence of mutation in the target epitope, and minimal shedding or extracellular secretion are the most important properties that an ideal mAb target should have. However, B-cell surface proteins identified to date do not concomitantly display all of these features. In addition, the relative contribution of the mechanisms responsible for the observed anti-tumour activity still remains largely unknown as well as the events leading to tumour resistance, which currently represents one of the major limitation of mAb therapy (Davis, Czerwinski et al. 1999; Cartron, Watier et al. 2004; Zhou, Hu et al. 2008). Therefore, novel Ab-based immunotherapeutics continue to be under evaluation to improve the current therapies for B-cell malignancies.

The strategy to conjugate mAbs with toxin(s) (immunotoxin(s)) has been developed to more efficiently target rapidly modulating antigens and induce tumour-cell death. However, this approach has been limited by the induction of immune responses to the mAb and the toxin as well as non-specific toxicity (Messmann, Vitetta et al. 2000). This has led to the generation of agents with an improved safety profile. The calicheamicin-conjugated anti-CD22 mAb Inotuzumab ozogamicin (CMC-544) is such an example (DiJoseph, Armellino et al. 2004) that, having demonstrated significant single-agent activity in relapsed FL and DLBCL (Fayad, Patel et al. 2006), is now under clinical investigation in combination with rituximab (Fayad, Patel et al. 2008) (Table 1.6).

Table 1.6 Clinical efficacy of mAb therapy for B-cell lymphomas.

Agent	Type	Description	Target	Status	Dose	Patients	Results	
							ORR (%)	CR (%)
Ofatumomab (HuMax-CD20)	mAb	Human	CD20	Phase I/II	300-1000 mg/m2/wk (x4)	Relapsed/refractory FL (n=38)	63	
Ofatumomab (HuMax-CD20)	mAb	Human	CD20	Phase I/II	300 mg (dose 1), 500 or 1000 mg (dose 2-8)/m2/wk (x8)	Rituximab refractory FL (n=116)	10	1
Ofatumomab (HuMax-CD20)	mAb	Human	CD20	Phase I/II	100-2000 mg/m2/wk (x4)	Relapsed/refractory CLL (n=33)	44	
Ofatumomab (HuMax-CD20)	mAb	Human	CD20	Phase II	300 mg (dose 1), 1000 mg (dose 1-8)/m2/wk (x8)	Relapsed/refractory DLBCL (n=81)	11	4
Ofatumomab (HuMax-CD20)	mAb	Human	CD20	Phase III	300 mg (dose 1), 2000 mg (dose 2-12) /m2/wk (x8) & /mos (x4)	Refractory CLL (interim analysis n=138)	58	
Veltuzumab (hA20)	mAb	Humanized	CD20	Phase I/II	80-750 mg/m2/wk (x4)	Relapsed/refractory B-NHLs (n=82)	40	21
GA101	mAb	Humanized	CD20	Phase II	1600 mg (d1 and d8); 800 mg (d22, q21) (x8)	Relapsed/refractory indolent NHLs (n=22)	55	9
GA101	mAb	Humanized	CD20	Phase II	1600 mg (d1 and d8); 800 mg (d22, q21) (x9)	Relapsed/refractory aggressive NHLs (n=19)	32	
Epratuzumab	mAb	Humanized mLL2	CD22	Phase I/II	120-1000 mg/m2/wk (x4)	Relapsed/refractory FL (n=55)	18	6
Epratuzumab	mAb	Humanized mLL2	CD22	Phase I/II	120-1000 mg/m2/wk (x4)	Relapsed/refractory aggressive lymphomas (n=56)	10	5
Epratuzumab Rituximab	mAb mAb	Humanized mLL2 Mouse-Chimeric	CD22 CD20	Phase II	360 mg/m2/wk (x4) 375 mg/m2/2wk (x4)	Relapsed/refractory FL (n=41)	54	24
Epratuzumab Rituximab	mAb mAb	Humanized mLL2 Mouse-Chimeric	CD22 CD20	Phase II	360 mg/m2/wk (x4) & /2mos (x4) 375 mg/m2/2wk (x4) & /2mos (x4)	Untreated FL (n=60)	84	33
Epratuzumab Rituximab CHOP	mAb mAb	Humanized mLL2 Mouse-Chimeric	CD22 CD20	Phase II	360 mg/m2/wk (x4) 375 mg/m2/2wk (x4)	Untreated DLBCL	95	72

Inotuzumab ozogamicin (CMC-544)	ADC (calicheamicin conjugated)	Humanized	CD22	Phase I	1.8 mg/m2, every 28 days	Relapsed/refractory FL or DLBCL (n=34)	53	24
Inotuzumab ozogamicin (CMC-544)	ADC (calicheamicin conjugated)	Humanized	CD22	Phase I	1.8 mg/m2, every 28 days	Relapsed/refractory FL (n=13)	85	54
Inotuzumab ozogamicin (CMC-544) Rituximab	ADC (calicheamicin conjugated) mAb	Humanized Mouse-Chimeric	CD22 CD20	Phase I/II	1.8 mg/m2 on day 2, every 28 days	Relapsed/refractory FL or DLBCL (n=30)	80	43
Inotuzumab ozogamicin (CMC-544)	ADC (calicheamicin conjugated)	Humanized	CD22	Phase II	1.8 mg/m2 on day 2, every 28 days	Rituximab relapsed/refractory indolent NHLs (n=43)	53	19
Lumiliximab FCR	mAb	Primate-Chimeric	CD23	Phase I/II	375 mg/m2 (n = 3) or 500 mg/m2 (n = 28)	Relapsed/refractory CLL (n=31)	65	52
Dacetuzumab (SGN-40)	mAb, mild agonist	Humanized	CD40	Phase I	2-8 mg/kg/wk (x6)	Relapsed/refractory B-NHLs (n=50)	12	2
Galiximab	mAb	Primate-Chimeric	CD80	Phase I/II	500 mg/m2/wk (x4)	Relapsed/refractory FL (n=38)	11	5
Galiximab Rituximab	mAb mAb	Primate-Chimeric Mouse-Chimeric	CD80 CD20	Phase II	500 mg/m2/wk (x4) 375 mg/m2/2wk (x4)	Relapsed/refractory FL (n=64)	64	17
Galiximab Rituximab	mAb mAb	Primate-Chimeric Mouse-Chimeric	CD80 CD20	Phase II	500 mg/m2/wk (x4) & /2mos (x4) 375 mg/m2/2wk (x4)	Untreated FL (n=61)	70	38
Galiximab Rituximab	mAb mAb	Primate-Chimeric Mouse-Chimeric	CD80 CD20	Phase II	500 mg/m2/wk (x4) 375 mg/m2/2wk (x4)	Relapsed/refractory FL (n=337)	51	20

Mos: months; wk: week; ORR: objective response rate; CR: complete response.

In contrast to unmodified mAb and drug/toxin mAb conjugates, tumour-specific mAbs complexed to a radioisotope (131I, 90Y) do not need to bind to each tumour cell, or penetrate homogeneously into the neoplastic tissue to exert their effects. The ionizing energy emitted from therapeutic isotopes can kill cells several cell diameters away (cross fire effect), thus resulting in a more efficient tumour targeting. Two radiolabelled mAbs directed against CD20 have been tested for the treatment of relapsed/refractory NHLs after first-line chemotherapy: 131I-tositumomab (Bexxar) and 90Y-ibritumomab tiuxetan (Zevalin) (Emmanouilides 2003; Vitolo, Ferreri et al. 2008). Both have provided high response rate in large cohorts of patients with relapsed/refractory FL (Witzig, Flinn et al. 2002; Witzig, White et al. 2003; Fisher, Kaminski et al. 2005; Kaminski, Radford et al. 2005). 90Y-ibritumomab showed also greater efficacy than rituximab as single-agent in a randomized trial (Witzig, Gordon et al. 2002). For the majority of patients, however, the response duration is relatively short and, at present, it is difficult to predict the best candidates for this treatment.

Therefore, the need for more cost-effective immunotherapeutics has led to the development of novel scaffold strategies, many of which are based on the use of mAb fragments or single-domain Abs. The aim is to increase target multispecificity, an increased ability to recruit effector cells, and to bind cryptic epitopes.

Small-modular immunopharmaceuticals (SMIPs) are single-chain polypeptides with a target-binding domain attached to an effector domain through a flexible hinge domain, the latter designed to govern the engagement of receptors on immune cells for enhanced ADCC activity (Gill and Damle 2006). TRU-015 and TRU-016 are recently developed CD20- and CD37-SMIPs that have demonstrated more potent ADCC than rituximab in CLL and NHLs. Ongoing research is investigating the potential clinical use of these SMIPs in B-cell lymphomas (Burge, Bookbinder et al. 2008; Hayden-Ledbetter, Cerveny et al. 2009) (Table 1.5).

Bispecific T-cell engager (BiTE) Abs that consist of two single-chain Abs, one specific for CD3, a subunit of T-cell receptor complex, and the other for a tumour-associated antigen, currently represent the most promising novel immunotherapeutics (Brischwein, Parr et al. 2007). Blinatumomab (MT103/MEDI538;bscCD3xCD19) is the first BiTE Ab tested clinically in patients with relapsed NHLs that has provided excellent anti-tumour activity in patients with FL, mantle-cell lymphoma, and CLL in a recent phase-I clinical trial (Bargou, Leo et al. 2008). Ongoing phase-II studies will clarify the role of this compound for the treatment of B-cell malignancies.

1.2.2 Active immunotherapy

The presence of a specific humoral and /or cellular immunity against the autologous malignancy has been found to be associated with a significant improved survival (Zhang, Conejo-Garcia et al. 2003; Goodell, Salazar et al. 2006; Al-Shibli, Donnem et al. 2008; Hornychova, Melichar et al. 2008). Thus, many strategies have been developed to actively generate such immunity in cancer patients to ameliorate their prognosis. The major advantage of active anti-tumour immunotherapy is the potential to stimulate an endogenous immune response against immunogenic proteins involved in malignant transformations, which, through the initiation of a tumour-specific long-term immunologic memory, may be able to contain the disease and prevent relapse.

The possibility of reactivating the host defensive functions against cancer was firstly recognized by William Coley in patients with established tumours that experienced spontaneous regression after pathogen infections. Starting with these observations, he applied the first nonspecific immunostimulatory approaches, based on the inoculation of bacterial material, leading to the concept of therapeutic vaccination against cancer (Coley 1891).

Thanks to expanding genetic and proteomic technologies, a large number of human cancer antigens has now been identified, paving the way for the development of specific vaccines (Rosenberg 1999). Tumour antigens have been broadly classified into two categories: shared self or tumour-associated antigens (TAAs) and unique or tumour-specific antigens (products of mutated genes, altered cell surface glycolipids and glycoproteins or viral antigens) (Wang and Rosenberg 1999). Shared antigens refer to proteins that are inappropriately expressed by tumour cells but can be found also in normal tissues (tyrosinase, alphafetoprotein (AFP), carcinoembryonic antigen (CEA), cancer-testis antigens, human epidermal growth factor receptor 2 (HER2)/neu protein). Unique tumour antigens result from mutations induced through physical, chemical carcinogens or from viral infection, and they are, therefore, expressed only by certain tumour types or even individual tumours (BCR-ABL, prostate-specific antigen (PSA), Ig-Id, EBV-EBNAs and- latent membrane proteins (LMPs), HPV-E6/E7).

Since stimulating the immune response against self-antigens has the potential to cause autoimmunity, the best way to attain robust protective anti-tumour immunity is most likely driven by breaking the tolerance to unique cancer antigenicities (Baurain, Colau et al. 2000; Echchakir, Mami-Chouaib et al. 2001; Karanikas, Colau et al. 2001; Schamel, Arechaga et al. 2005; Sensi, Nicolini et al. 2005). Thus, optimally designed cancer vaccines should combine the most adequate immunostimulatory agents and delivery strategies in order to provide the efficient *in-vivo* cross-presentation of the most tumour-specific antigens by the most potent APCs (DCs) to T cells. Such an approach is expected to enhance the probability of inducing a clinically successful immune response associated with a specific long-lasting immunological memory that can keep in check the outgrowth of tumour metastases, thus prevening relapses (Figure 1.10).

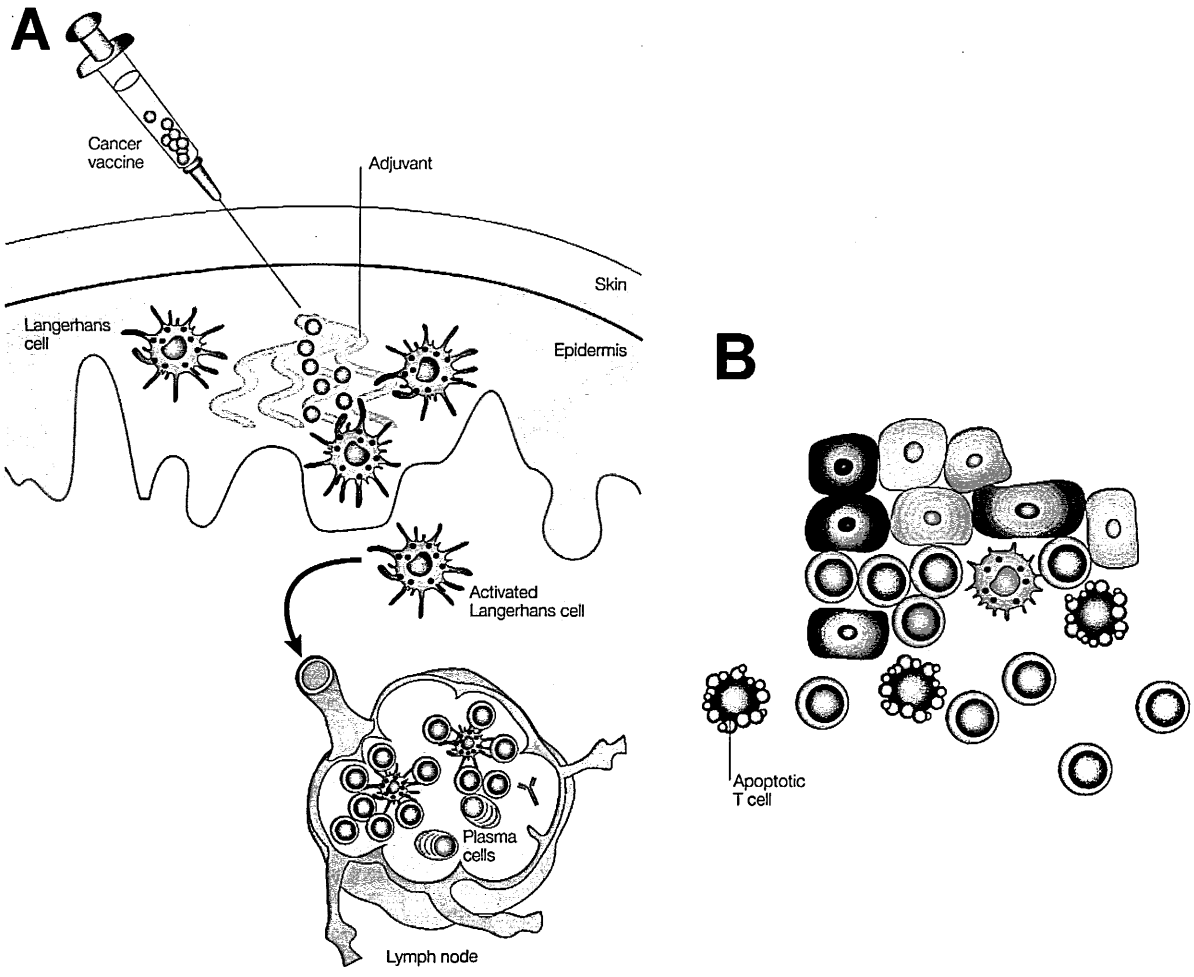


Figure 1.10 Therapeutic anticancer vaccines.

In the optimal clinical setting, therapeutic vaccines intend to boost immunity against minimal residual disease and prevent the outgrowth of metastases. (A) Vaccines are administered with adjuvant agents that favour the activation of Th1 specific responses through the stimulation/recruitment of proper DCs and T cell subsets. Tumour antigens can be taken up by activated DCs (such as epidermis-resident Langherans cells) that, trafficking to the draining lymph node, present them to T cells, leading to the clonal expansion of tumour-specific T cells and the production tumour specific Abs. (B) Tumour-specific T cells migrate to the site of tumour metastases where they attempt to kill tumour cells that express antigens contained in the vaccine. (Adapted from Finn 2003)

Recognition of the central role of DCs has allowed the classification of anticancer vaccine into two categories depending on the tumour antigen delivery (Figure 1.11):

1. *In-vivo* DC therapy, which uses whole tumour cells, proteins, peptides, RNA, DNA, viral vectors antigens linked to DC maturation stimuli for *in-vivo* antigen uptake by DCs in a pro-inflammatory environment;
2. *Ex-vivo* DC therapy, which uses *ex-vivo* generated DCs loaded with whole tumour cells, proteins, peptides, RNA, DNA for the stimulation of CTLs upon injection into patients.

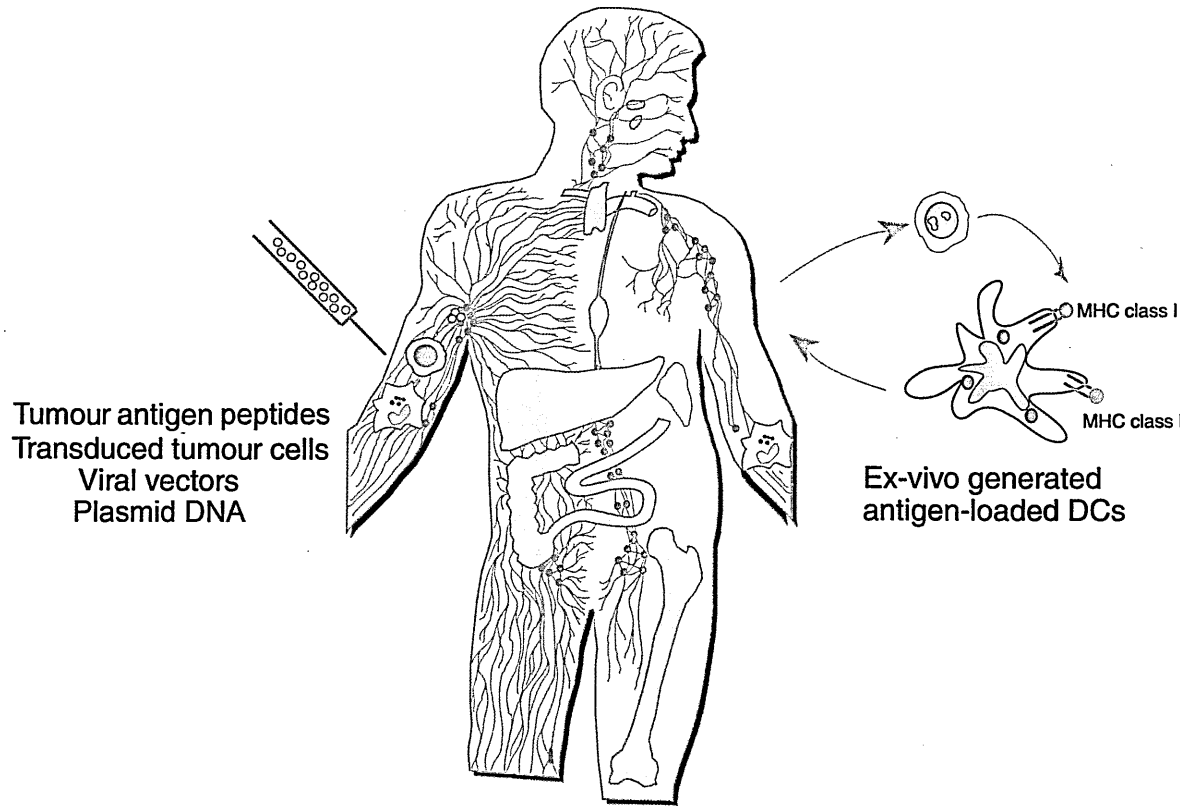


Figure 1.11 In-vivo and ex-vivo DC therapy.
Left: injection of the immunogenic agents (viral vector or plasmid DNA encoding for the tumour antigen, or tumour cells transduced to express the antigen, or antigen peptides) for a random target of DCs *in vivo*. Right: injection of *ex-vivo* generated tumour antigen-loaded DCs. (Adapted from Palucka 2007)

On the basis of the successes of attenuated pathogen vaccines to treat viral diseases and owing to the initial lack of defined tumour antigens, whole killed tumour cells or tumour cell lysate represented the initial forms of anticancer vaccines (Ward, Casey et al. 2002). Upon injection, tumour cells are destroyed as a consequence of the inflammatory reaction or spontaneous apoptosis induction, and antigens can be picked up by DCs and cross-presented to T cells for the priming of a specific adaptive immune response.

Thereafter the strategy to use whole tumour cells (Harris, Ryan et al. 2000; Kusumoto, Umeda et al. 2001; Jocham, Richter et al. 2004), or allogeneic cell lines for a less cumbersome procedure (Toes, Blom et al. 1996; Jaffee, Hruban et al. 2001; Sosman, Unger

et al. 2002), has continued to be exploited due to the main advantage of eliciting a specific polyclonal immune response without the need to *a priori* know the tumour antigens. Accordingly, tumour cells have been also genetically modified to increase their immunogenicity and be used as a vaccine (Jaffee, Hruban et al. 2001; Kusumoto, Umeda et al. 2001).

The discovery of TAAs laid the basis for the design of anticancer vaccines specifically targeting tumour cells. In this field, peptide- or protein-based vaccines have been the most frequently formats used. Recently, synthetic or naturally occurring polypeptides encompassing a variety of immunogenic CTL and T-helper (Th) epitopes has provided a significant improvement in the immunogenicity of peptide-based vaccines. These peptides not only enable immune responses against more than one tumour antigen, but also favour the concurrent stimulation of CD4+ Th cells needed to induce and sustain the MHC class I-restricted CD8+ T-cell activation (Fayolle, Deriaud et al. 1991; Shirai, Pendleton et al. 1994; Hiranuma, Tamaki et al. 1999; Zeng, Li et al. 2002). Such long polypeptides are taken up and processed by specialized APC rather than binding directly to MHC class I molecules (as do peptides that are 8 to 10 amino acids long) offering an interesting opportunity for the induction of robust anti-tumour responses (Melief and van der Burg 2008). Tumour Id, the unique determinants of malignant B-cell Ig, has been the most widely exploited antigen as anticancer vaccines in B-cell lymphoma. Originally generated by hybridoma technology, anti-Id protein can now be produced by recombinant technology cloning Ig genes into stable cell lines for its use as a vaccine. To date, several TAAs, for which T cells of a cancer patients and, sometimes, healthy individuals have shown reactivity, have been tested in clinical trials. However, as yet, no a single TAA-based vaccine has gained official drug approval owing to lack of proven clinical efficacy (Claesson 2009).

As an additional option to target DC *in vivo* and to immunize cancer patients, viral vectors and plasmid DNA encoding TAAs have been exploited. This strategy requires *in-vivo* transfection and antigen production. The optimized gene sequence is delivered intradermally, subcutaneously (Gene Gun device), or to the muscle (intramuscular injection – and electroporation), which allows, respectively, the transfection of professional APC (epidermal keratinocytes and Langherans DCs) or myocytes and secondary cross-presentation of tumour antigens by recruited DCs. Plasmid DNA vaccines are highly flexible, since they can encode several immunological components to direct and amplify immunity with low cytotoxicity, are relatively stable and potentially cost-effective for manufacture and storage. Viral-mediated gene transfer is more disadvantageous because of the immunogenicity of the viral packaging proteins, their high expense, toxic side effects, limits on transgene size, and potential for insertional mutagenesis (Hacein-Bey-Abina, Von Kalle et al. 2003). Initial clinical trials in lymphoma (Hawkins, Zhu et al. 1994; Timmerman, Singh et al. 2002) melanoma (Tagawa, Lee et al. 2003; Weber, Boswell et al. 2008), prostate (Low, Mander et al. 2009; McNeel, Dunphy et al. 2009), and cervical (Kim, Gambhira et al. 2008) carcinomas have demonstrated the safety and immunological efficacy of DNA-based vaccines, with no relevant levels of integration into host cellular DNA, or the development of anti-nuclear auto-Abs. However, one note of caution is the possibility that vaccine antigen uptake and presentation may take place in the improper DC subset without the adequate stimuli, thus resulting in tolerance or an unwanted type of immunity rather than in the priming of an anti-tumour adaptive immune response (Palucka, Ueno et al. 2007).

By contrast, when generated *ex-vivo*, DCs are properly matured starting from CD34+ hematopoietic progenitors or more commonly from peripheral blood (PB)-derived monocytes (Banchereau and Palucka 2005; Nestle, Farkas et al. 2005). Following incubation with a cocktail of maturation cytokines (Sallusto and Lanzavecchia 1994), they are loaded with tumour antigens as to recapitulate *ex vivo* the early phase of DC activation.

However, variables associated with employing DC vaccines are numerous and require precise consideration for their therapeutic efficacy. They include the source or DC lineage to use, the antigen engulfing strategy, the DC maturation and/or activation levels to achieve *ex-vivo* and the route of vaccine administration. Physiological activation of DCs occurs in two phases, maturation and licensing, both of which are essential steps enabling antigen-loaded DCs to migrate to the draining lymph nodes (LNs) where they can activate T cells against the antigens they are presenting. DC licensing should be avoided during their *ex-vivo* generation for vaccine purpose, since *ex-vivo* DC full activation may lead to the impairment of their immunogenic function *in vivo*. The goal, therefore, is to differentiate antigen-loaded DCs only to the point that they have acquired LN migratory capacity and become responsive to licensing stimuli *in vivo* when they reach the LN and encounter cognate T cells (Gilboa 2007).

The form of antigens used to load DCs is a crucial aspect to consider when designing anticancer vaccination strategies, since it affects the major histocompatibility complex (MHC) antigen presentation pathways and thereby the induction of cytotoxic CD8+ and/or helper CD4+ T cell responses (Nestle, Farkas et al. 2005). The feasibility of using MHC class I or II peptides has been a field of intense interest. However, although the peptides can be easily chemically synthesized under Good Manufacturing Practice (GMP) requirements, these logistical advantages are offset by the paucity of known tumour-specific peptides (especially MHC class II restricted peptides), the limited stability of exogenous peptide-MHC complexes and patient-specific MHC haplotype restriction. Use of protein-based polyepitopic antigens to load DCs, which allows peptides to be channelled into both the MHC class I and MHC class II presentation pathways, is limited by access to clinical grade reagents. Generating clinical-grade libraries of overlapping peptides is more feasible than generating clinical-grade proteins but is expensive, and regulatory organizations might prevent the use of this approach if each peptide component has to be validated separately. Therefore loading DCs with total antigens, such as recombinant proteins, dying tumour cells, and exosomes, or transducing DCs with viral vectors and mRNA (Jenne, Schuler et al. 2001; Gilboa and Vieweg 2004) seems to be the most promising strategy, since it allows the natural processing and epitope selection improving the efficacy of antigen presentation. Transfection of DCs with tumour transcriptome has turned out to be a more efficient method to load DCs (Gilboa and Vieweg 2004) compared to the use of tumour cell-derived cDNA (Van Tendeloo, Ponsaerts et al. 2001). Unfortunately, DC transfection has the disadvantage of channelling tumour-specific antigens primarily into the MHC class I presentation pathway, thus limiting the generation of effective CD4+ T cell responses (Yewdell, Norbury et al. 1999). By contrast, DCs have been shown to efficiently process phagocytosed dead tumour cells generating peptides for both CD8+ and CD4+ T cell-specific recognition (Fujii, Fujimoto et al. 1999; Palucka, Ueno et al. 2006). The use of whole-tumour antigens therefore has the advantage to simultaneously deliver many TAAs (known and unknown, including unique TAA that are expressed by individual tumour) and generate CTL responses across all MHC classes, eliminating the need to predetermine the most suitable antigens that in a vaccine to mediate tumour rejection. In particular, these vaccine approaches have shown to activate CTLs against specific TAAs, as carcinoembryonic antigen (CEA) (Nair, Boczkowski et al. 1998), prostate-specific antigen (PSA) (Heiser, Dahm et al. 2000), telomerase reverse transcriptase (TERT) (Nair, Heiser et al. 2000), and Id (Di Nicola, Zappasodi et al. 2009). In addition, under certain conditions, dying tumour cells may provide intrinsic maturation signals for DCs through the release of heat-shock proteins (HSP)90, and HSP70 and other molecular chaperones, such as gp96, calreticulin (CRT) (Basu, Binder et al. 2000). It is worth to consider, however, that the advantages of whole tumour cell-based vaccines of widening the spectra of TAAs against which eliciting an immune response is counterbalanced by the potential risk of autoimmune reactions to non-tumour self-antigens (Finn 2003).

Finally, the administration route of antigen-loaded DCs has been also recognized to be a critical factor for the induction of a specific cellular immune response. Although intranodal injection has shown to be the most efficient way to provide DC accumulation in secondary lymphoid organs (Lambert, Gibson et al. 2001), it is still unknown whether overloading this tissue with large amount of injected DCs really improve the occurrence of immunological and clinical responses in humans (Lesimple, Neidhard et al. 2006). Therefore, subcutaneously or intradermally injected DCs, which showed to reach the regional LNs and favour the onset of a protective immunological memory in contrast to those delivered intravenously, have been the most widely used (Fong, Brockstedt et al. 2001; Mullins, Sheasley et al. 2003).

The recognition of the low immunogenicity of cancer antigens and the resultant low avidity of specific T cells, together with the immunosuppressive network established by tumour cells, has spurred the development of combination strategies to overcome these limitations and improve the efficacy of cancer vaccines. Matzinger's "Danger Model" pioneered the idea that tumour antigens presented in the context of bacterial signals may be viewed by the immune system as 'dangerous' leading to tumour elimination by the immune system (Matzinger 2002). Modifiers of the innate immunity for shaping the proper adaptive immune response have been thus considered essential components of anticancer vaccines. Accordingly, the co-administration of adjuvant agents has been largely exploited in active immunotherapy protocols, with the molecules mostly used being Freund's adjuvant, keyhole limpet hemocyanin (KLH) (Harris and Markl 2000), QS21, ISCOMs, montanide, HSPs, bacilli Calmette-Guerin (BCG), granulocyte/monocyte-colony stimulating factor (GM-CSF) (Higano, Corman et al. 2008), toll like receptor (TLR) ligands (Imiquimod for TLR-7/8, CpG for TLR-9) (Goldstein, Varghese et al. ; Datta, Cho et al. 2004; Li, Song et al. 2007; Brody, Ai et al. 2010). The progressive understanding of homeostatic mechanisms that control strength and duration of an immune response has led to the use of reagents that block the down-regulation or enhance the stimulation of anti-tumour immune activation. Numerous studies have shown that antagonistic Abs specific for co-inhibitory receptors, such as cytotoxic T lymphocytes-associated antigen 4 (CTLA-4) (Hodi, Mihm et al. 2003; Peggs, Quezada et al. 2006) and PD-1 (Korman, Peggs et al. 2006; Keir, Francisco et al. 2007), or regulatory cytokines, such as tumour growth factor-beta (TGF-β) (Gorelik and Flavell 2001) and interleukin (IL)-10 (Moore, de Waal Malefyt et al. 2001), Treg inactivation (Ruter, Barnett et al. 2009) as well as enhancement of DC-stimulation through Abs targeting CD205 (DEC205), the mannose receptor, DC-SIGN, Langerin, and CD40 (Tacken, de Vries et al. 2007) allow for sustaining the proper anti-tumour adaptive immunity in response to a cancer vaccine towards a clinical efficacy. An additional valuable mechanism that can be potentiated by these strategies is the ability of some vaccines to generate epitope spreading through the stimulation of the immune system against off-target antigens, thus avoiding selective pressure of tumour antigen loss variant and immunoediting, and eliciting a polyepitopic immune response associated with clinical benefit (Disis, Bernhard et al. 2009).

1.2.2.1 Evaluation of immune response to active immunotherapy

There is a general agreement that immune monitoring of vaccinated patients should guide the development of cancer vaccines (Keilholz, Martus et al. 2006), since an accurate measurement of immune biomarkers can allow determining whether a tumour specific immune response can lead to tumour regression. Robert Koch described the host's delayed-type hypersensitivity (DTH) reaction following the intradermal injection of inactivated poxvirus as the first surrogate marker for specific immunity and protection (Koch 1890; Hogrefe 2005). Since then, a variety of *in-vitro* assays have been developed to measure immune responses to vaccines including proliferation tests (initially ^{3}H thymidine

incorporation assay and more recently carboxyfluorescein diacetate succinimidyl ester (CFSE) dilution assay), killer cell function tests (initially ^{51}chromium release assay and more recently flow cytometry-based cytotoxic assay using CFSE or PKH26 and 7-AAD co-staining), and the most recent tests of antigen specificity (tetramer analysis, Enzyme-linked immunosorbent spot (ELISPOT)) and intra-cellular cytokine flow cytometry (CFC)).

Peptide-MHC I and II tetrameric complexes (tetramers) allow the enumeration, concurrent phenotyping, characterization, and isolation of peptide specific CD8+ and CD4+ T cells. The limit of detection of tetramers binding T cells is 1 tetramer-binding cell per 8,000 cells. The main limitation of this method stems from the rare event analysis required to detect cell-binding tetramers (Hogrefe 2005).

ELISPOT assay, developed in 1983, recognizes antigen-dependent T-cell release of a particular cytokine, such as interferon gamma (IFN-γ), using plates coated with the specific "capture" mAb and a second enzyme-labelled Ab directed to a different epitope of the same cytokine. Specific cytokine release by a T cell upon antigen recognition is revealed as spots that are counted by a computer. In the 1990s, ELISPOT was shown to correlate with the standard chromium-release cell-mediated cytotoxicity assay to measure T cell effector function. Antigen reactive cells are typically detectable at a limit of 1: 50,000 with this method. The magnitude of the response that may be associated with clinical efficacy remains unclear (Hogrefe 2005).

Like the ELISPOT, CFC assesses cytokine responses of individual cells but using intracellular staining with specific Abs, with a detection level of 1 antigen-reactive cell in 50,000. CFC is unique in its ability to define the T-cell subpopulations that responds to the vaccine by concurrently looking at the cell surface and intracellular markers (Hogrefe 2005).

These analyses alone, however, are not completely exhaustive, since they can reveal only whether T cells recognizing the antigen of interest have been stimulated. Functional tests that analyse whether these T cells can also destroy tumour cells are required. Surrogate biomarkers for the induction of a specific immunity after vaccination against cancer have been mostly developed measuring T-cell functions, since T-cell adaptive response has been always considered the *conditio sine qua non* for cancer regression after therapeutic vaccination.

Given the successes of mAbs therapies, however, humoral response to anti-tumour vaccination are being re-evaluated. It can be monitored in the sera of vaccinated patients by enzyme-linked immunosorbent assay (ELISA) (Weng, Czerwinski et al. 2004; Mander, Chowdhury et al. 2009), magnetic beads with immobilized antigen (Schmittling, Archer et al. 2008) or by flow cytometry and western blotting analyses using, as target, cells expressing the immunogenic proteins contained in the vaccines (Reuschenbach, {von Knebel Doeberitz} et al. 2009), or more recently protein arrays (Belousov, Kuprash et al. 2010).

In addition, there is a general agreement that combinations of multiple markers which do not only evaluate the quantity and quality of the elicited immune responses, but also host and tumour microenvironmental factors that may be critical in mediating a clinical response, will be most likely needed to evaluate the efficacy of immune based interventions as well as the optimal frequency and/or duration of treatment (Dang and Disis 2009).

1.2.2.2 Clinical benefit of active immunotherapy

To date, an overall objective response rate of 3.3% was observed in 1306 vaccine treatments of cancer patients with advanced disease (Claesson 2009). Patient responses to *ex-vivo* DC vaccinations were higher (9.5%) than those to anti-tumour immunotherapies using peptides (2.7%), viral vectors (1.9%), or irradiated tumour cells (4.6%) (Banchereau and Palucka 2005) (Table 1.7, for representative examples).

Table 1.7 Clinical efficacy of anticancer vaccines.

Vaccine type	Cancer type	Vaccine	Total patients	Patients responding
Peptide	Melanoma	Tyrosinase + GMCSF	16	0
	Melanoma	Peptides in IFA or on DC	26	3
	Melanoma	MART-1 + IL-12	28	2
	Prostate	Peptides	10	0
	Melanoma	Peptides on PBMC + IL-12	20	2
	Breast and prostate	Telomerase	7	0
	Cervix	HPV16 E7	17	0
	Colorectal	Peptides in IFA	10	0
	Multiple	NY-ESO-1	12	0
	Multiple	Ras in DETOX adjuvant	15	0
	Multiple	Peptides in IFA	14	0
Virus	Prostate	Vaccinia-PSA	33	0
	Prostate	Vaccinia-PSA	42	0
	Colorectal	Vaccinia-CEA	20	0
	Colorectal	Vaccinia-CEA and B7-1	18	0
	Multiple	Avipox-CEA(IGMCSF)	60	0
	Multiple	Avipox-CEA	15	0
	Multiple	Vaccinia + avipox-CEA	18	0
Tumour cells	Melanoma	Transduced with GM- CSF	26	1
	Melanoma	Membranes on silicone beads	17	1
	Lung	Transduced with GMCSF	26	1
	Lung	Transduced with GMCSF	43	3
	Breast	Transduced with B7-1	30	0
Dendritic cells	Melanoma	Pulsed with peptides	17	0
	Melanoma	Pulsed with peptides or lysates	33	3
	Melanoma	Pulsed with peptides or lysates	16	5
	Melanoma	Pulsed with peptides	24	1
	Melanoma	Pulsed with MAGE-3A1 peptide	11	0
	Childhood cancers	Pulsed with lysates	15	1
	Kidney	Transfected with RNA	15	0
	Colorectal	Pulsed with CEA peptides	12	1
	Kidney	Pulsed with tumour lysates	35	3
	Multiple	Pulsed with tumour lysates	20	0
Heat shock protein	Melanoma	grp96	28	2
	Multiple	grp96	16	0
		Total	765	29

(Adapted from Rosenberg 2004)

Despite the limited clinical efficacy of therapeutic cancer vaccines, many such cancer vaccine trials have been optimistically reported because surrogate or subjective endpoints, such as histological evidence of tumour necrosis or lymphocyte infiltration, were achieved. Sensitive techniques, such as tetramer or ELISPOT assays, have been used

to demonstrate the *in-vivo* generation of anti-tumour T cells in vaccinated patients, but the paucity of clinical responses in these patients has made it difficult to validate any of these assays as useful surrogate of clinical efficacy. Little and/or inconsistent correlations have been shown between immunological and clinical responses in immunized cancer patients, being concordant (Banchereau, Palucka et al. 2001; Belli, Testori et al. 2002; Mazzaferro, Coppa et al. 2003), discordant (Lee, Wang et al. 2001; Vonderheide, Domchek et al. 2004) or even mutually exclusive (Rosenberg, Sherry et al. 2005), depending upon the trial. Noteworthy, the results obtained from circulating T cells may not reflect what is happening within the tissue itself.

The fact that vaccine trials have been performed in heavily pre-treated patients with progressive refractory tumours may be one of the reason of the limited clinical efficacy of cancer vaccines, since the immunosuppressive effects of the tumour microenvironment greatly impair the development of an effective anti-tumour immunity (Finn 2003). Considering that an evolving immune response could most likely prevent rather than treat disease relapse, an active immunotherapeutic intervention has been regarded more appropriate after disease stabilization following the gold-standard treatment or as an adjuvant therapy. Unfortunately, several phase-III trials that aimed at definitely proving the clinical benefit of anticancer vaccines in a wide number of patients with limited malignant disease, including melanoma, lymphoma, colon, prostate and renal carcinoma, failed to clearly demonstrated the primary end-point of an increased disease/progression free survival (Harris, Ryan et al. 2000; Hersey, Coates et al. 2002; Mitchell, Abrams et al. 2007) (Morton, Mozzillo et al. 2007; G-VAX, available from: http://www.fiercebiotech.com/story/deaths-force-halt-cellgenesys-cancer-vaccine-trial/2008-08-27; OncoPhage, available from: http://www.fiercebiotech.com/press-releases/fda-grantspermission-export-antigenics-oncophage-russia; MyVAX, FavId).

To date, autologous DCs pulsed *ex vivo* with prostate acid phosphatese (PAP) has been the first anticancer vaccine that achieved the regulatory approval by the FDA, upon the demonstration of a median survival improvement of 4.1 months and a death risk reduction of 22.5% in vaccinated hormone-refractory prostate cancer patients compared to the control group (Kantoff, Higano et al. 2010). Surprisingly, the prolongation of survival was not found to be associated with a measurable anti-tumour effect, raising concern that an imbalanced study-group assignment, caused by unmeasured or unknown prognostic variables, might have influenced the results (Kantoff, Higano et al. 2010).

There is now a general interest towards the understanding of the failure and the few successes of anticancer vaccines in order to develop improved approaches and properly design their evaluation in clinical studies. Until now, the limited efficacy of cancer vaccines to induce the expansion of high levels of anti-tumour T cells able to infiltrate the tumour and become activated after encountering the specific antigen *in vivo* is one of the main reasons for their inability to definitely clear solid tumours (Rosenberg, Yang et al. 2004). Lymphoid malignancies allow an easier access to the circulation and often express costimulatory molecules for the activation of naïve and memory cells, thus partially explaining their slightly increased sensitivity to DC vaccines (Timmerman 2002). Several studies have demonstrated that large numbers of adequately activated, tumour-specific T cells can mediate the rejection of large, vascularised tumours in mice and humans (Rosenberg, Sherry et al. 2005). Cancer vaccines most likely need to generate these levels of immune activation to reach clinical efficacy. To increase the chance for responsiveness and to limit the risk of vaccine-induced immunoediting and TAA down-modulation, the use of polyepitopic vaccines, including the real "tumour-regression" antigens that demonstrated to induce immune activation associated with clinical responses in cancer patients, seems theoretically the most promising approach (Claesson 2009). In addition, agents able to break tolerance and stimulate the proliferation of anti-tumour lymphocyte

into patients without autoimmune toxicity will likely be required to enhance vaccine effects.

A leading example of the apparently inconsistent successes and failure of active immunotherapy for cancer patients is the experience of Id vaccines. After two decades spent on the validation of this approach in preclinical models (Lynch, Graff et al. 1972; Kaminski, Kitamura et al. 1987; Kwak, Young et al. 1996) as well as in initial clinical trials (Inogès, Rodrìguez-Calvillo et al. 2006) in 2008 two randomized clinical trials aimed at confirming these proof-of-principles in a large-scale setting failed (Park and Neelapu 2008; Freedman, Neelapu et al. 2009), and, although a third study showed encouraging results in this respect, it has not lead to regulatory approval for the marketing of an Id-based vaccine owing to insufficient patient accrual and statistical significance(de Cerio and Inoges 2009) (Table 1.8).

Table 1.8 Main features and interpretation of clinical trials assessing the clinical benefit of Id vaccines for FL.

	University of Navarra, Spain	Genitope	Favrille	NCI/Biovest
Vaccine	generic	MyVax	FavId	BiovaxId
Patients	FL, first relapse	FL, untreated	FL, untreated or relapsed	FL, untreated
Source of tumour	Excisional biopsy	FNA/core biopsy	FNA/core biopsy	Excisional biopsy
Idiotype production	Heterohybridoma technology	Recombinant DNA technology	Recombinant DNA technology	Heterohybridoma technology
Induction therapy	Six cycles of CHOP or CNOP	CVP (8 cycles efery 3 wk)	Rituximab (weekly x 4)	PACE/R-CHOP (6-8 cycles every 4wk)
Comparison (experimental/control)	Same patients: second compared with first CR	2/1 randomization	1/1 randomization	2/1 randomization
Patient status before vaccination	Second CR	First CR or PR	First CR, PR or SD	First CR or CRu
Vaccination	Id-KLH+GM-CFSE (sc, 23-30 mo of active vaccination)	Id-KLH+GM-CFSE or KLH+GM-CSF (sc, 7 doses)	Id-KLH+GM-CFSE or placebo+GM-CSF (sc, until PD)	Id-KLH+GM-CFSE or KLH+GM-CSF (sc, 5 doses)
Number of patients (actual/expected)	25/ 25	Vaccine: 192/240; control: 95/120	Vaccine: 174 instead of 171; control: 175 instead of 171	Vaccine: 76/250; control: 41/125
Primary end point	Second CR > first CR and 13 months	PFS ($p<0.01$)	TTP ($p<0.01$)	DFS ($p<0.01$)
Results	20 of 20 immune responders achieved both end points; 5 of 5 immune non-responders did not	Median PFS, 19.1 (experimental) vs 23.3 (control) mo ($p = 0.297$)	Median TTP, 9 (experimental) vs 12.6 (control) mo ($p = 0.019$)	Median DFS, 44.2 (experimental) vs 30.6 (control) mo ($p = 0.045$)
Potential caveats	Small proof-of-principle study	More patients than expected (at least one-quarter) relapsed or progressed within 9 months from randomization	Too few CR induction before vaccination; severe B cell depletion by rituximab in most vaccinated patients	Missed target ITT and statistically significant
Refs	Inoges et al. 2006	Levy et al. 2009	Freedman et al. 2009	Schuster et al. 2009

CHOP, cyclophosphamide, doxorubicin, vincristine and prednisone; CNOP, cyclophosphamide, mitoxantrone, vincristine and prednisone; RTX: Rituximab; CRu, complete response unconfirmed; CVP, cyclophosphamide, vincristine and prednisone; DFS, disease-free survival; GM-CSF, granulocyte-macrophage colony-stimulating factor; ITT, intent to treat; KLH, keyhole limpet haemocyanin; n.s, not significant; PACE, prednisone, doxorubicin, cyclophosphamide and etoposide; PFS, progression-free survival; pts: patients; CR, complete response; PR, partial response; SD, stable disease; TTP, time to progression. (Adapted from Bendandi 2009)

The formal proof that KLH-conjugated Id plus GM-CSF can function as a tumour specific vaccine leading to immune response and rejection of the corresponding tumour was provided by preclinical vaccination-challenge studies using murine model of myeloma and lymphoma (Lynch, Graff et al. 1972; Kaminski, Kitamura et al. 1987; Kwak, Young et al. 1996). The immune responses were highly heterogeneous and therapeutically efficacious

when the tumour size was limited, indicating that the best clinical setting in which to evaluate the clinical effects of Id vaccination was represented by patients in complete response after standard chemotherapy (Kaminski, Kitamura et al. 1987). The biological efficacy of vaccination with Id-KLH plus GM-CSF was subsequently demonstrated by most phase I/II studies in FL patients in complete clinical response or with residual disease after chemotherapy (Bendandi, Gocke et al. 1999; Inogès, Rodrìguez-Calvillo et al. 2006; Redfern, Guthrie et al. 2006; Yanez, Barrios et al. 2008). Only one Phase II study showed the association between the occurrence of an Id-specific immune activation and the clinical benefit in terms of an improvement in relapse-free survival in vaccinated FL patients (Table 1.8) (Inogès, Rodrìguez-Calvillo et al. 2006). Therefore, 3 large-scale randomized phase-III clinical trials were initiated with the aim of demonstrating a clear-cut survival improvement in vaccinated patients. These studies used either hybridoma-derived Id (BiovaxId, Biovest International, Schuster et al. 2009) or recombinant Id (MyVax, Genitope, (Levy et al. 2008); FavId, Favrille, (Freedman, Neelapu et al. 2009)) in grade 1-3 FL patients experiencing at least disease stabilization (Favrille), partial (Genitope) or complete (Biovest) remission after standard course of rituximab, CVP or PACE (prednisone, doxorubicin, cyclophosphamide and etoposide) regimens, respectively (Table 1.8). Although these clinical trials failed to confirm the clinical benefit of Id vaccination, they also did not prove that the negative results were due to the failure of the vaccine rather than to defects in study design. The pre-vaccine treatment modality has been recognized as one of the major flaw of these studies: rituximab-treated patients started vaccination with severely depleted numbers of circulating B cells, CVP did not always produce durable response, and PACE regimen proved inappropriate in the rituximab era, thus explaining the very limited patient enrolment and randomization. In addition, given the heterogeneous (i) natural history of indolent NHLs, (ii) influence of these tumours on host defences (Bendandi 2009), (iii) responsiveness of these patients to the Id-antigen (Lynch, Graff et al. 1972), (iv) the anti-tumour effects of an immune response induced by Id-vaccines (Inogès, Rodrìguez-Calvillo et al. 2006), coupled with the possible emergence of target antigen mutation or down-regulation over the course of the disease (Meeker, Lowder et al. 1985; Raffeld, Neckers et al. 1985), the use of standard phase-III randomized trials does not seem the most appropriate to assess the clinical benefit of patient-specific Id-vaccines (Bendandi 2006; Longo 2006). The study of Id-vaccination in lymphoma patients in first relapse and/or progression after chemotherapy would allow the possibility to compare the length of the clinical responses obtained with the subsequent treatments (standard chemotherapy vs. vaccination protocol). In this regard, a longer duration of the vaccine-induced second clinical response compared to the first in a substantial proportion of patients has been proposed as the major end-point for regulatory approval without facing the specific complications of a randomization (Longo 2006; Bendandi 2009). Randomization to either idiotypic vaccination or maintenance rituximab after chemotherapy has been also suggested with the non-inferiority of Id vaccines compared to rituximab as a second independent main clinical end point, possibly allowing regulatory approval (Longo 2006).

1.2.3 Adoptive Cell Transfer Immunotherapy

Due to their high cytotoxic potential, specific activity and efficient homing function, T cells have been considered the major immune actors in the fight against cancer (Mitchison 1955). Several immunotherapeutic strategies have aimed to take advantage of T cells using specific mAbs activating or blocking respectively co-stimulatory or co-inhibitory molecules, and/or, as previously described, their *in-vivo* targeting with anti-tumour vaccination. Alternatively, the infusion of tumour-reactive autologous or allogeneic T cells into cancer patients as adoptive cell transfer therapy (ACT) has been exploited to augment

anti-tumour immunity above the levels provided by vaccination alone and thus increase the possibility to eradicate established diseases.

The direct evidence that anti-tumour T cells can induce a specific response associated with clinical benefit is shown by the durable complete remissions observed in many individuals with haematological malignancies treated with DLI upon relapse following allogeneic transplantation (Dazzi, Szydlo et al. 2000; Lokhorst, Schattenberg et al. 2000). These effects have been ascribed to a graft-versus-tumour effect of donor T cells that can recognize in the recipient a pattern of HLA containing TAA-derived peptides as non-self (Cooper, Topp et al. 2003; Porter and Antin 2006; Sprangers, Van Wijmeersch et al. 2007). The ability of allogeneic HSCT or DLI to eradicate tumour and provide long-term survival, even in highly chemotherapy-refractory lymphomas, demonstrated the susceptibility of these diseases to T cell-mediated immune responses (Khouri, McLaughlin et al. 2008; Rezvani, Storer et al. 2008). Although potentially curative, the treatment-associated non-relapse mortality and chronic GVHD limit the application of these therapeutic interventions. In addition, they cannot be used in many patients because of advanced age and co-morbidities as well as the unavailability of a suitable donor. Therefore, efforts have been made to generate T cells targeting tumour antigens minimally expressed in normal tissues for harnessing this anti-tumour effector activity avoiding the toxicity of allogeneic HSCT and DLI. The generation of tumour-specific T cells involves many variables, including the source of T cells, the strategy to achieve anti-tumour T cell specificity, the methods of T cell stimulation and expansion, all of which affect dramatically the success of ACT (Table 1.9).

Table 1.9 Variables to consider in the generation of anti-tumour T cells for adoptive transfer.

Starting cell type	Method of stimulation	Exogenous cytokines	Timing of restimulation
Unsorted PBMC	Phytohemagglutinin	IL-2	Weekly
CD8+ T cells	anti-CD3 Ab	IL-2 + IL-15	Every 10-14 days
CD4+ T cells	anti-CD3 and -CD28 Abs	IL-15	By size
CD8+ and CD4+	anti-CD3/-CD28 beads	IL-7 + IL-15	When target cells die
Central memory T cells	Irradiated target cells	IL-12	
Effector memory T cells	Irradiated aAPC	IL-21	
Naive T cells	Irradiated aAPC expressing co-stimulatory ligands	Other combinations	

(Adapted from Till and Press 2009)

The finding that tumour infiltrating lymphocytes (TILs) could be expanded in the presence of IL-2 whilst still retaining the MHC-restricted recognition of the autologous tumour cells represented a leading step in the development of tumour specific ACT (Muul, Spiess et al. 1987). The infusion of *in-vitro* expanded and stimulated autologous TIL in metastatic melanoma patients represented the first example of ACT for the treatment of

advanced solid cancer (Figure 1.12) (Rosenberg, Yannelli et al. 1994; Dudley, Wunderlich et al. 2002; Dudley, Wunderlich et al. 2005).

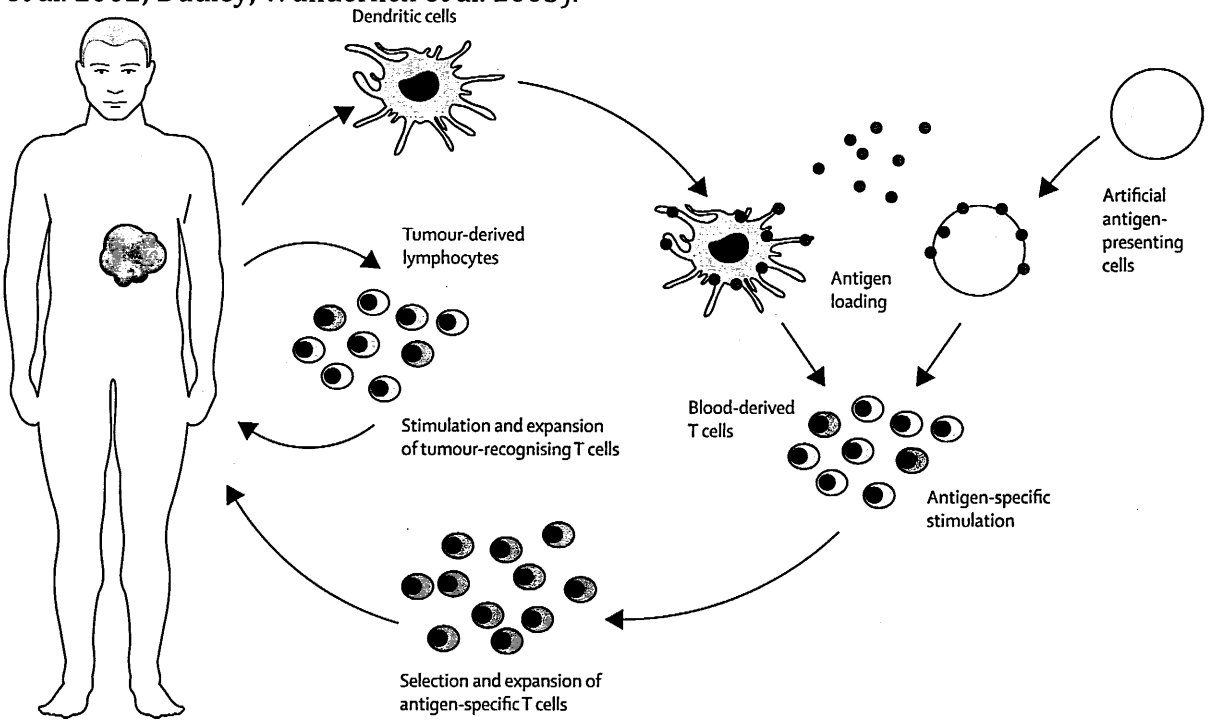


Figure 1.12 Isolation and ex-vivo stimulation of antigen-specific T cells for adoptive transfer.
Autologous PB or tumour-derived T cells are expanded ex vivo in the presence or not of TAA-loaded APCs, such as dendritic cells or artificial APCs. Enriched tumour-specific T cells are then re-infused for treatment. (Adapted from Disis 2009)

However, initial attempts to treat cancer patients with *ex-vivo* stimulated TILs met with limited success, largely due to the inability to fully restore adequate effector functions in autologous TILs *in vitro* (Kolenko, Wang et al. 1997; Radoja, Saio et al. 2001). The finding that autologous PB-derived T cells could be more easily rescued by anergy *in vitro* (Ohlen, Kalos et al. 2001; Teague, Sather et al. 2006) led to start using this cell population for the generation of tumour specific T cells by repetitive *ex-vivo* stimulation in the presence of professional APC loaded with the antigen(s) of interest (Figure 1.12) (Riddell and Greenberg 1990). Using this approach, however, the magnitude and persistence of the transferred immunity have been limited, mainly because T-cell cloning needs a long culture period and preferentially induces the differentiation of T cells with an effector-memory phenotype (T_{EM} cells). In contrast to initial beliefs, fully matured and activated effector T cells have shown limited anti-tumour efficacy *in vivo*, mainly because of their inability to be further stimulated *in vivo* to lyse tumour cells and persist within the memory compartment (Figure 1.13) (Yee, Thompson et al. 2002; Gattinoni, Klebanoff et al. 2005; Hinrichs, Borman et al. 2009).

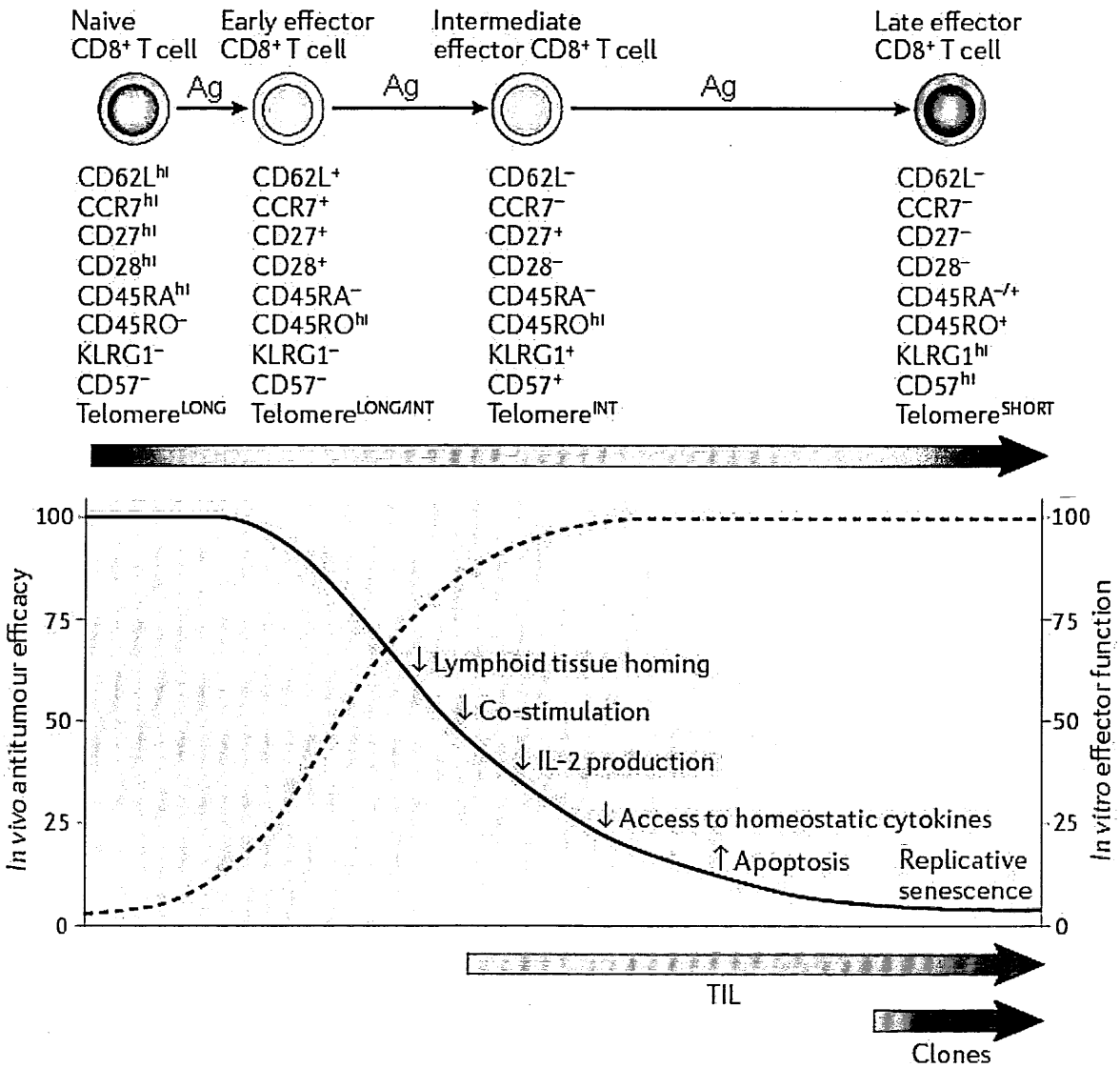


Figure 1.13 Inverse relationship of in vitro and in vivo anti-tumour functions of adoptively transferred naive and effector T-cell subsets.

Chronic or repetitive antigen stimulation drives naive CD8+ T cells to a terminally differentiated effector state and ultimately to exhaustion. For each step that characterizes this process the phenotypic changes are illustrated. Together with a shortening of the telomere length, CD8+ T cells progressively lose their proliferative potential and become exhausted and/or undergo apoptosis. In addition, CD8+ T cells lose the ability to secrete IL-2 and to respond to homeostatic cytokines such as IL-7 and IL-15. Although *in-vitro* tests have revealed the progressive gain of effector functions and lytic capability with stimulation, highly *in-vitro* reactive and cytolytic tumour-specific CD8+ T-cell clones loose their ability to mediate cancer regression when adoptively transferred. Ag: antigen. (Adapted from Gattinoni 2006)

Studies aimed at defining T-cell characteristics that are crucial for a clinically efficacious ACT intervention have indeed identified their *in-vivo* persistence and tumour homing properties as the most relevant (Schwartzentruber, Hom et al. 1994; Robbins, Dudley et al. 2004; Zhou, Shen et al. 2005). These findings have highlighted the need for strategies that are able to expand more undifferentiated T cells (i.e. expressing CD62L/CCR7 molecules) that can be further activated in situ in the host LNs, thus promoting the onset of long-lasting therapeutic anti-tumour effects into patients. Although potentially useful to reduce the length of *in-vitro* antigen-specific T cell stimulation whilst preserving their destructive functions, the rapid *in-vitro* expansion of TAA-specific T cells generated *in vivo* by specific immunisation has rarely been investigated (Schultze, Anderson et al. 2001; Rapoport, Stadtmauer et al. 2005; Powell, Dudley et al. 2006). Since

multiple observations have indicated the ability of polyclonal T cells generated by *ex-vivo* short expansion from bulk peripheral blood mononuclear cell (PBMC) cultures to efficiently mediate tumour rejection (Yee, Thompson et al. 2002; Antony, Piccirillo et al. 2005; Dudley, Yang et al. 2008; Muranski and Restifo 2009), it has been now realized that the mixture of naïve, effector, T_{EM} and central memory T cells (T_{CM}), of both CD4+ and CD8+ T cells (recognizing multiple TAAs and having long-lasting *in-vivo* persistence potential), may represent a more effective T-cell product for ACT compared to highly selected specific CD8+ T cell clones (Antony, Piccirillo et al. 2005). Besides classic "helper" function, CD4+ T cells have been reported to directly destroy tumour cells and/or recruit and activate other immune cells, in particular APCs (Corthay, Skovseth et al. 2005; Perez-Diez, Joncker et al. 2007; Hunder, Wallen et al. 2008). New approaches to polyclonal T-cell expansion are currently attempting to replace autologous DCs by more efficient artificial APCs to shorten the *in-vitro* culture period whilst driving T-cell activation and maturation towards the most suitable phenotype to maximize T cell *in-vivo* persistence and tumour homing properties upon adoptive infusion (Figure 1.12) (Maus, Thomas et al. 2002; Oelke, Maus et al. 2003).

In addition, the possibility of the introduction of high affinity T-cell receptors (TCR) and co-stimulatory molecules into circulating human lymphocytes has more recently allowed redirecting T cell specificity towards TAAs present on multiple tumour types (Gross, Waks et al. 1989; Johnson, Heemskerk et al. 2006). TCRs, obtained from rare reactive human clones or from transgenic mouse cells following immunisation against human cancer antigens, are now available for a wide array of cancer antigens expressed by common solid and hematologic tumours (Heemskerk, Hoogeboom et al. 2004; Cohen, Zheng et al. 2005; Roszkowski, Lyons et al. 2005; Zhao, Zheng et al. 2005). ACT with genetically engineered T lymphocytes has demonstrated clinical efficacy against several malignancies (Morgan, Dudley et al. 2006).

To avoid the limitations of MHC restriction imposed by the use of αβ TCRs, chimeric TCRs, in which the variable regions of an Ab are genetically fused to an intracellular T- or B-cell signalling domains (most commonly CD3ζ, chain), have been developed against a variety of targets, including HER2 (Stancovski, Schindler et al. 1993), CEA (Haynes, Trapani et al. 2002), MART1, MAGE-A1 (Clay, Custer et al. 1999), prostate specific membrane antigen (PSMA) (Ma, Safar et al. 2004) and CD33 (Finney, Lawson et al. 1998). Chimeric TCRs have proven to efficiently redirect the recognition specificity of lymphocytes to cell surface TAAs in initial clinical studies (Park, Digiusto et al. 2007). Since the clinical effects of ACT with chimeric TCRs bearing T cells were very modest, second generation chimeric TCRs, incorporating co-stimulatory molecule signalling domains, have been developed. They resulted in strong antigen-dependent activation and expansion of transfected T cells, leading to the design of a series of new phase-I clinical trials (Bendle, Haanen et al. 2009).
As there are many variables to consider in the generation and expansion of T cells, there are also many potential manipulations of the recipient that can be done to maximize the *in-vivo* activity of the modified T cells. The strategy to apply conditioning regimens prior to ACT has been shown to create a niche for the enhancement of infused T cell homeostatic proliferation (Tan, Ernst et al. 2002; Gattinoni, Finkelstein et al. 2005) by depleting immunosuppressive cells (Antony, Piccirillo et al. 2005; Sakaguchi 2005) as well as normal endogenous lymphocytes that act as competitor cells for homeostatic cytokines (Figure 1.14) (Dummer, Niethammer et al. 2002). Furthermore, by killing tumour cells, systemic chemotherapy and total body irradiation boost TAA release and cross-presentation, thus facilitating the activation of transferred tumour-reactive T cells (Kedl, Rees et al. 2000) (Figure 1.14).

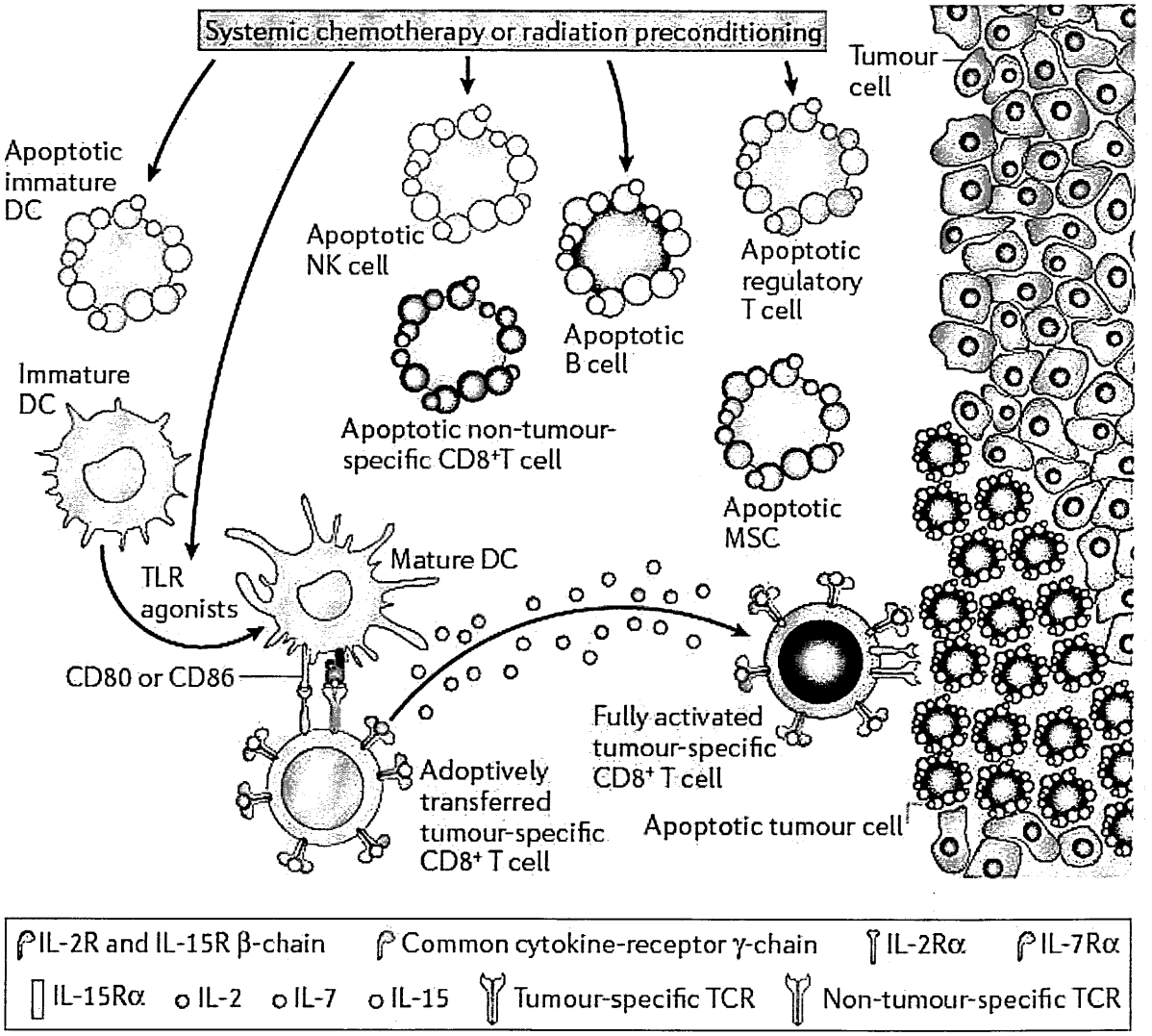


Figure 1.14 Mechanisms underlying the impact of lymphodepletion on adoptively transferred T cells. Systemic chemotherapy or radiation before ACT might modify the tumour-bearing host. Although APCs are reduced in number by direct killing, the reduced competition for antigen at the APC surfaces may result in a net increase in lymphocyte activation. The release of (Toll like receptor) TLR agonists after mucosal damage favours DCs maturation and lymphocyte activation. Due to the removal of host lymphocytes, NK cells, Tregs and myeloid-derived suppressor cells (MDSCs), activating cytokines, such as IL-2, IL-7 and IL-15, may be increasingly available and the host immunosuppression activities are decreased. These modifications may explain the improved anti-tumour efficacy of transferred CD8+ T cells observed in lymphodepleted hosts (Adapted from Gattinoni 2006).

Recent adoptive transfer trials for melanoma using antigen-specific T cells following aggressive lymphodepletion regimens have shown considerable tumour regression sometimes associated with long periods of T-cell survival in patients with refractory metastatic disease (Dudley, Wunderlich et al. 2005; Morgan, Dudley et al. 2006; Dudley, Yang et al. 2008). To prolong the *in-vivo* survival of transferred T cells even further, cytokines has been co-administered with ACT as adjuvant therapy. The initial use of high-dose IL-2 (Yee, Thompson et al. 2002; Dudley, Wunderlich et al. 2005; Till, Jensen et al. 2008) is being gradually replaced by other cytokines, such as IL-7 and IL-15, since exogenously administered IL-2 has been found to also favour the expansion of Tregs (Melchionda, Fry et al. 2005).

The successes of ACT in melanoma have spurred the application of this strategy for the treatment of other malignant diseases, including B-NHLs, on the basis of their high susceptibility to immunotherapeutic approaches and their expression of suitable antigens to be targeted with specific T cells. The development of post-transplant

lymphoproliferative diseases (PTLD), caused by the reactivation of EBV infection in B cells of donor or recipient origin after allogeneic HSCT or solid organ transplants, respectively, continue to be a significant clinical problem (Rosenberg, Restifo et al. 2008). The adoptive transfer of *in-vitro* generated EBV-specific allogeneic T-cell lines have shown to efficiently treat and/or prevent PTLD in allogeneic HSCT (Rooney, Smith et al. 1998) and solid organ transplant patients (Haque, Wilkie et al. 2002; Haque, Wilkie et al. 2007). Unlike PTLD, in which the immunocompromised state of the host allows the expression of immunodominant EBV epitopes, EBV-related nasopharyngeal carcinoma (NPC) (Comoli, Pedrazzoli et al. 2005) and Hodgkin lymphoma (Bollard, Aguilar et al. 2004) express only the weakly immunogenic proteins of latent EBV (LMP1, LMP2 and EBNA-1) (Bollard, Cooper et al. 2008). In these cases, ACT with LMP-1 and LMP-2 specific T cells have achieved increased clinical efficacy compared to polyclonal anti-EBV cell lines; however, the time required for their generation makes the procedure not suitable for the treatment of patients with active disease (Bollard, Gottschalk et al. 2007).

Antigen-specific TCR-bearing T cells have also been generated for the treatment of NHLs. As for mAb therapy, the most widely exploited lymphoma-associated biotargets for transgenic TCR have been CD20 and CD19. Anti-CD20 chimeric TCR-bearing T cells have shown to expand in bulk culture from transfected autologous PBMCs and their use in ACT has resulted safe, feasible, and a well-tolerated treatment. However, the clinical responses were modest, probably due to the low surface expression of the transfected TCR, and the low *in-vivo* persistence of transferred T cells due to the protracted expansion time (Jensen, Cooper et al. 2003; Wang, Press et al. 2004; Till, Jensen et al. 2008). T cells expressing chimeric anti-CD19 TCR can be generated and expanded from lymphoma patients (Cheadle, Gilham et al. 2005) and are now under investigation as ACT in clinical trials. Meanwhile, the incorporation of co-stimulatory molecule (CD28, ICOS, 4-1BB) signalling domains in second-generation anti-CD19 and anti–CD20 chimeric TCRs have already shown to enhance Th1 cytokine secretion by stimulated T cells, their *in-vivo* persistence and anti-tumour effects in recent pre-clinical studies (Imai, Mihara et al. 2004; Kowolik, Topp et al. 2006; Milone, Fish et al. 2009).

Cell transfer studies have thus demonstrated that the administration of highly avid anti-tumour T cells directed against a suitable target in a lymphodepleted host environment can mediate the regression of certain large, vascularised, metastatic cancers in humans. Their use has provided guiding principles as well as encouragement to further develop immunotherapy for the treatment of cancer patients. However, several limitations currently hamper the wide application of ACT, as listed below:

I. Not all tumour-specific T cell cultures can be sufficiently expanded to reach the amount needed for a therapeutically efficacious ACT;
II. Not all tumour-specific T cell cultures can be optimized at killing the tumour upon infusion into patients;
III. The *in-vivo* infusion of large amount of *ex-vivo* activated and expanded lymphocytes entails important safety concerns, particularly when they are genetically modified;
IV. The patient-specific nature of anti-tumour ACT makes the procedure very costly and cumbersome and cannot be easily transferred to the commercial-scale mass-production techniques necessary to reach the multitude of cancer patients worldwide.

Whilst new insights and innovations in the coming years may overcome many current limitations, optimization of the presently known variables of ACT programmes might improve their therapeutic application.

1.2.4 Comprehensive immunotherapy

Comprehensive immunotherapy refers to an integrative cancer medicine that not only focuses on the neoplastic cells but also and with the same importance, on the regulatory, repair and immune mechanisms of the cancer patient as a whole. This concept was re-evaluated since its first proposal about fifty years ago by Dr Josef M. Issels (ISSELS J, 1970). It is now generally accepted that the intimate interplay between cells of the immune system and cancer is ongoing from the initiation of oncogenesis, and thus suppressive or repair mechanisms represent an intrinsic phenomenon of cancer (de Visser, Eichten et al. 2006). Anticancer immunotherapy should thus address the multifactorial nature of the disease using combination protocols that, whilst promoting tumour shrinkage by multiple fronts, can enhance the persistence of anti-tumour immune responses, T cell co-stimulation, DC immunogenicity, and counteract tumour immune evasions mechanisms.

The few past years has seen as many improvements in the field of immunotherapy as in the development of novel anticancer drugs, leading to the possibility of a range of combinatorial therapeutic options. The mechanism of action of older and newer anticancer therapies has become increasingly clear, and the list of conventional and unconventional compounds that can modulate the host environment is rapidly growing (Zitvogel, Apetoh et al. 2008). Accumulating evidence about the immunoadjuvant side effects of radiotherapy and several chemotherapeutic compounds has recently opened new possibilities for the design of rationale chemo-immunotherapy combination strategies able to directly kill tumour cells, whilst activating the immune system to produce a more robust and sustainable anti-tumour response. The concept of combining immune therapy with sequential or even concurrent chemotherapy, previously considered counterintuitive due to the intrinsic immune toxicity of any kinds of chemo- and radiotherapy, is now being re-considered. Indeed, data from several studies have revealed that combining immunotherapy and chemotherapy can favour the onset of clinical responses in advanced cancer patients (Wheeler, Das et al. 2004; Gribben, Ryan et al. 2005; Arlen, Gulley et al. 2006; Laheru, Lutz et al. 2008; Ramakrishnan, Antonia et al. 2008). Conventional therapies have been found to promote the activation of the immune system against cancer cells in two principal ways: as on-target effects, they can confer an increased immunogenic potential to tumour cells, thus enhancing cancer susceptibility to a specific immune attack, (van der Most, Currie et al. 2008); as off-target effects, they can directly stimulate or inhibit, respectively, immune effector or regulatory cells (Ghiringhelli, Menard et al. 2007), or influence stroma cells (Dudley, Yang et al. 2008).

Table 1.10 Immunostimulatory properties of cytotoxic drugs.

Bioactivity	Mechanisms	Drugs
Antigen uptake	Cell-surface CRT	Anthracyclines, irradiation
Antigen processing	Increased MHC class I expression	Irradiation, DAC
	Increased TAA production	Irradiation, DAC
	mTOR activation	Irradiation
	HMGB1 release	Anthracyclines, oxaliplatin‡, irradiation
Antigen presentation	DC activation (through TLR4 and MyD88)	Paclitaxel§ (in mice), irradiation
	APC activation (through TBK, IRF3 and IFN)	Vascular-disrupting agents
	HSP90	Bortezomib‖
	Self-peptide–MHC class I complexes	Myelosuppression and HSCs
	Increased antigen cross-presentation	Gemcitabine¶
T-cell-dependent anti-tumour effects	T-cell activation	Taxanes and intratumour DCs or GM-CSF-containing vaccines
		Anthracyclines, oxaliplatin, irradiation
		IL-12 with DAC
		5-fluorouracil with vaccines
Homeostatic proliferation	Cytokine sink removal?	Low doses of total body irradiation
	TReg-cell inhibition?	High doses of alkylating agents, fludarabine
	Microbial translocation?	
Homing to tumours	Increased ICAM1, PECAM and VCAM1 expression	Irradiation
	Increased RANTES and CXCL10 production	Vascular-disrupting agents
Target destruction	Increased CD95 expression	Cisplatin, irradiation, 5-fluorouracil and dacarbazine
	Increased HSP production	5-Fluorouracil
	Decreased succinate dehydrogenase activity	Cisplatin
	Increased TRAIL expression	Imatinib mesylate# with IL-2
Generation of memory T cells	Homeostatic proliferation	Total body irradiation
	Self-peptide–MHC class I complexes	Cyclophosphamide, fludarabine with or without vaccines or HSCs
Inhibition of Tregs	Inhibition of MSCs and B cells	Gemcitabine
	Inhibition of TReg cells	Cyclophosphamide
	Inhibition of M2 macrophages	Taxanes
	Reduced TGFbeta production	Bleomycin
NK cell triggering	DC–NK-cell crosstalk	Imatinib mesylate
	Type I IFNs	Vascular-disrupting agents

‡Eloxatin; Sanofi–Aventis; §Taxol; Bristol–Myers Squibb; ||Velcade; Millennium Pharmaceuticals; ¶Gemzar; Eli Lilly; #Gleevec, Novartis; APC, antigen-presenting cell; CXCL10, CXC-chemokine ligand 10; DAC, 5-aza-2'-deoxycitidine; DC, dendritic cell; GM-CSF, granulocyte/macrophage colony-stimulating factor; HMGB1, high-mobility group box 1 protein; HSC, haematopoietic stem cell; HSP, heat-shock protein; ICAM1, intercellular adhesion molecule 1; IFN, interferon; IL, interleukin; IRF3, IFN-regulatory factor 3; MSCs, myeloid suppressor cells; mTOR, mammalian target of rapamycin; NK, natural killer; PECAM, platelet/endothelial cell-adhesion molecule; TBK, TANK-binding kinase; TGFβ, transforming growth factor-beta; TLR4, Toll-like receptor 4; Treg cells, CD4+CD25+ regulatory T cells; TRAIL, tumour-necrosis-factor-related apoptosis-inducing ligand; VCAM1, vascular cell-adhesion molecule 1. (Adapted from Zitvogel 2008)

Based on initial observations by Albert and colleagues (Albert, Sauter et al. 1998), the biomolecular mechanisms through which apoptosis process may stimulaye the immune system against dying cells have been extensively studied and certain anticancer cytotoxic agents have shown a peculiar property to promote antitumour immune activation. These observations have demonstrated the possibility to immunize against cancer through its killing using cytoreductive treatments (Sauter, Albert et al. 2000; Zitvogel, Casares et al. 2004). Cytotoxic agents able to influence the immunogenicity of tumour cell death were identified by screening for the ability of treated tumour cells to generate protective immunity in immunocompetent syngeneic mice following an immunisation-challenge schedule (Obeid, Tesniere et al. 2007a; Obeid, Tesniere et al. 2007b). Using this strategy, anthracycline (doxorubicin (DXR), idarubicin, mitoxantrone), oxaliplatin and ionizing irradiation resulted the most potent inducers of immunogenic tumour cell death (Table 1.10). These agents showed the distinctive property of stimulating the rapid pre-apoptotic cell-surface translocation of the endoplasmic-resident chaperone calreticulin (CRT), which can promote DC phagocytosis and cross-presentation of TAAs to T cells, and, thus, the priming of a specific adaptive immune response (Obeid, Tesniere et al. 2007a; Obeid, Tesniere et al. 2007b)(Figure 1.15). As additional "eat-me" signals for DCs, the exposure of HSP70 and HSP90 on the plasma membrane has shown to contribute to the immunogenicity of dying tumour cells (Feng 2001; Binder and Srivastava 2005; Saito, Dai et al. 2005) (Figure 1.15). Accordingly, the proteasome inhibitor bortezomib (Velcade; Millennium Pharmaceuticals) and the antimetabolite drug 5- fluorouracil (5-FU) were found to boost the surface-translocation of HSP90 and HSP70, respectively, on human myeloma and gastric carcinoma cells, favouring their uptake by DCs and a specific T cell activation (Table 1.10) (Galetto, Buttiglieri et al. 2003; Spisek, Charalambous et al. 2007). As later immunogenic signals provided by dying cancer cells, the release of high-mobility group box 1 (HMGB-1), a ligand of TLR2 and TLR4 that facilitates TAA processing and cross-presentation by DCs (Scaffidi, Misteli et al. 2002; Apetoh, Ghiringhelli et al. 2007a; Apetoh, Ghiringhelli et al. 2007b), and adenosine triphosphate (ATP) (Ghiringhelli, Apetoh et al. 2009; Aymeric, Apetoh et al. 2010), which can promote the activation of DCs through the induction of inflammasome and the release of IL-1β, represent crucial events for the priming of a specific immune response associated with clinical benefit (Figure 1.15). It has been also becoming appreciated that treatment outcome is strongly influenced by characteristics of the individual patients and their counter-response to treatment. Importantly, the TLR4 Asp299Gly polymorphism, which was found to reduce the binding of HMGB1 to human TLR4, has been recently established as an independent predictive factor of early disease progression in metastatic colorectal and breast cancer patients treated with oxaliplatin- or anthracycline-based regimens, respectively (Apetoh, Ghiringhelli et al. 2007; Tesniere, Schlemmer et al. 2010). Similarly, the loss-of-function polymorphism Glu496Ala in the P2RX7 receptor, which severely delays the ATP-dependent IL-1β release (Gu, Zhang et al. 2001; Sluyter, Shemon et al. 2004), was found to exert a significant negative prognostic impact in breast cancer patients treated with anthracyclines (Ghiringhelli, Apetoh et al. 2009).

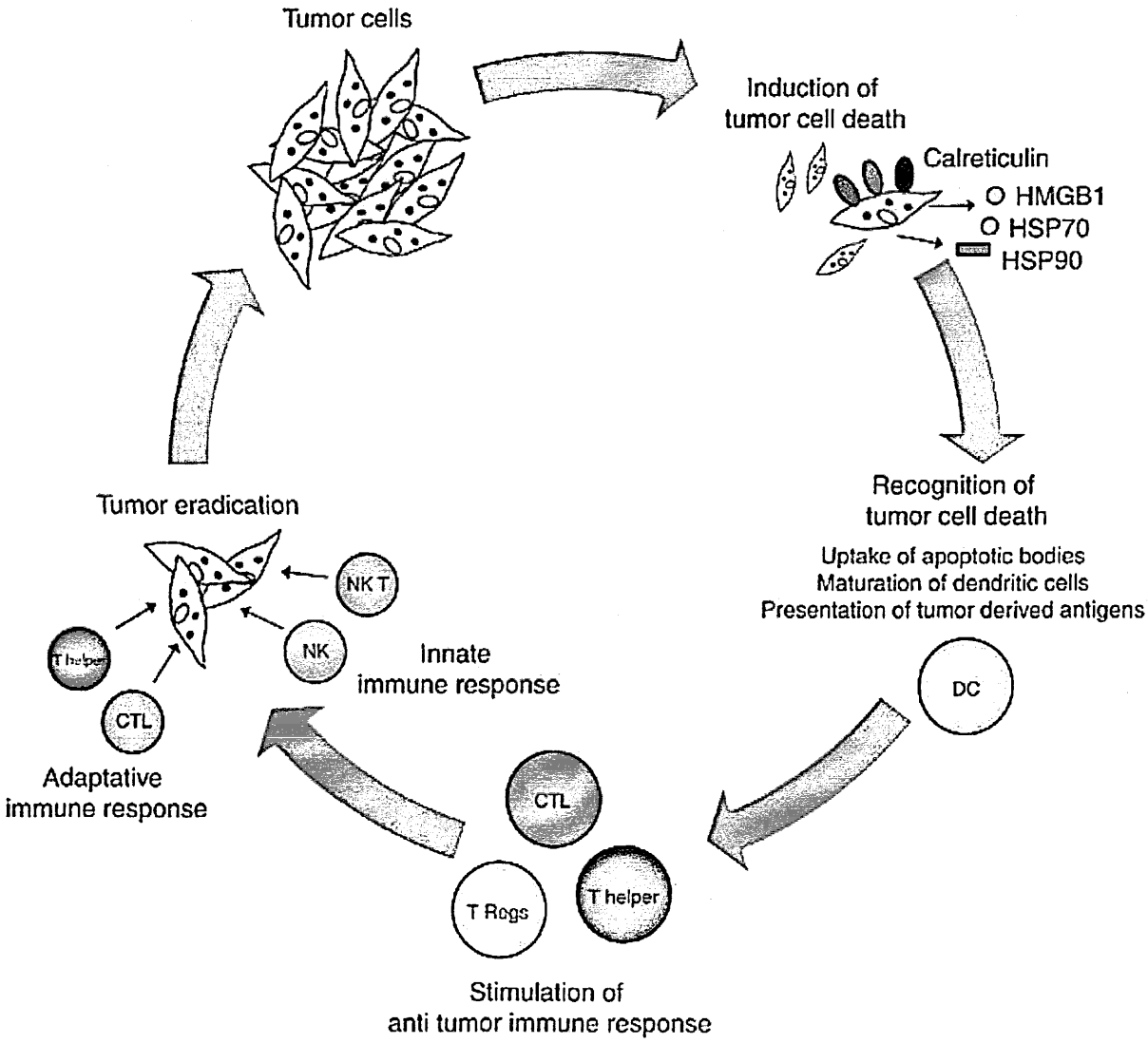


Figure 1.15 Steps leading to immunogenic tumour cell death.
Immunogenic cell-death response includes the rapid translocation of intracellular CRT to the cell surface, where it acts as a mandatory eat-me signal for DCs, followed by the appearance on the cell surface of other molecular chaperones, such as HSP90, that contribute to tumour cell– DC interaction and DC maturation. Then, dying cells release the chromatin-binding protein HMGB1 that is required for optimal TLR4-dependent processing of the phagocytic cargo by DCs. (Adapted from Tesniere 2008).

Beyond inducing immunogenic tumour cell death, some conventional cytotoxic agents may also render cancer cells more susceptible to killing by CTLs. For examples, 5-FU, irinotecan (CPT-11), cisplatin (CDDP), paclitaxel and DXR were shown to enhance lytic sensitivity of cancer cells by increasing their expression of co-stimulatory molecules, such as LFA-3 and ICAM-1 (Bergmann-Leitner and Abrams 2001; Wu, Zeng et al. 2007), or the mannose-6-phosphate (M6P) receptor, which is required for Granzyme B associated killing (Veugelers, Motyka et al. 2006; Ramakrishnan, Assudani et al. 2010) (Table 1.10). As part of its ability to modify transcription regulation in tumour cells, the DNA methyltransferase inhibitor 5- aza- 2′-deoxycitidine was shown to restore the expression of HLA class I molecules, predisposing neoplastic cells to immune attack (Serrano, Tanzarella et al. 2001; Kozar, Kaminski et al. 2003) (Table 1.10). Ionizing irradiation was also reported to up-regulate the expression of HLA class I molecules via the modulation of intracellular peptide repertoire, the stimulation of new protein synthesis and, thus, increased expression of tumour-derived antigenic peptides (Reits, Hodge et al. 2006), thereby boosting CTL activity (Garnett, Palena et al. 2004), and finally promote T cell trafficking towards irradiated

tumour sites (Table 1.10). "The abscopal effect", namely the reduction of distant non-irradiated metastases, represents the macroscopic effect of the profound anti-tumour immune modulation provided by irradiation (Munro 2009).

As off-target effects, conventional therapies may act on normal cells at the level of the tumour stroma or immune system, further promoting anti-tumour immune activation. A direct effect on immune cells has been demonstrated for most topoisomerase inhibitors and anti-microtubule agents that promoted DC maturation stimulating the secretion of IL-1β, IL-6 and IL-12p40 and/or inducing CD40, CD80, CD86 and MHC class II expression (Tanaka, Matsushima et al. 2009). For instance, taxanes were reported to enhance T- cell proliferation and NK- cell cytolysis in breast cancer patients (Carson, Shapiro et al. 2004). Another alkylating agent, dacarbazine, was shown to synergize with peptide vaccines in melanoma patients through the enhancement of memory CD8+ T-cell responses, highlighting the chemo-immunotherapeutic effects of this class of compounds (Nistico, Capone et al. 2009) (Table 1.10). The immunomodulatory drugs lenalidomide and pomalinomide have demonstrated clinical anti-tumour activity in several hematologic diseases, including CLL and indolent lymphoma subtypes (Witzig, Vose et al. 2007), which is related to their properties to inhibit pro-inflammatory cytokine production, increase NK cell cytotoxicity and regulate T-cell co-stimulation and activation (Ramsay, Clear et al. 2009) (Table 1.10).

The immunostimulatory properties of some chemotherapeutic compounds may also result from the inhibition of the immunosuppressive mechanisms promoted by the tumour. Gemcitabine, for example, facilitates the cross-presentation of TAAs by DCs, as a consequence of the direct cytotoxic effect on tumour cells, but it also showed to counteract the tumour-induced expansion of myeloid-derived suppressor cells (MDSCs) (Suzuki, Kapoor et al. 2005), favouring a clinically efficacious anti-tumour immunity when combined with recombinant cytokines or vaccines in patients with pancreatic (Plate, Plate et al. 2005), colon (Correale, Tagliaferri et al. 2008), or non small-cell lung cancer (NSCLC) (Levitt, Kassem et al. 2004) (Table 1.10). Correale and colleagues pioneered the field of chemoimmunotherapy in phase II trials for metastatic colon cancer by combining immunogenic chemotherapy (gemcitabine plus oxaliplatine) with GM-CSF and IL-2 (Correale, Cusi et al. 2005; Correale, Tagliaferri et al. 2008) and showed that tumour antigen-specific immune responses and autoimmune side effects can be associated with a positive clinical outcome (Correale, Del Vecchio et al. 2008). Continued administration of low ("metronomic") doses of cyclophosphamide was shown to selectively clear Tregs, inhibit their immunosuppressive function (Ghiringhelli, Menard et al. 2007), and stimulate IFN-α production (Schiavoni, Mattei et al. 2000), in agreement with previous clinical trials indicating the immunostimulatory function of this treatment (Berd, Maguire et al. 1986; Berd and Mastrangelo 1988; MacLean, Miles et al. 1996) (Table 1.10). Also taxanes, in particular paclitaxel and docetaxel, were found to reduce Treg frequency, either when administered alone (Zhang, Dermawan et al. 2008) or in combination with carboplatin (Wu, Feng et al. 2010) or cyclophosphamide (Tongu, Harashima et al. 2010) (Table 1.10). Similarly, preclinical studies have described the ability of lenalidomide and pomalinomide to inhibit Treg expansion and suppressor function (Galustian, Meyer et al. 2009).

Surprisingly, also targeted therapies, which most recently entered the clinical practice, partly exert their anti-tumour activity by immunoadjuvant side effects. For example, through the inhibition of c-KIT in host DCs, imatinib mesylate was shown to induce DC- mediated, NK cell activation, and DC-dependent anti-tumour effects in gastrointestinal stromal tumour (GIST) and chronic myeloid leukaemia (CML) patients (Borg, Terme et al. 2004) (Table 1.10). The level of IFN-γ secretion by NK cells after treatment with imatinib mesylate has been recognize as a positive prognostic parameter in patients with GIST, strengthening the clinical importance of the immunostimulatory effect of this drug (Menard, Blay et al. 2009). The novel multi-kinase inhibitors sunitinib,

successfully introduced in the treatment of patients with renal cell carcinoma (RCC), was reported to reduce the frequency of circulating Tregs and MDSCs (Ko, Zea et al. 2009), thus restoring T cells production of IFN-γ (Ozao-Choy, Ma et al. 2009).

The many-sided demonstration of the immune adjuvant effects of conventional anticancer therapies thus forms the basis upon which to initiate clinical trials combining chemotherapy and immunotherapy in a comprehensive strategy. Such an approach, whilst promoting tumour shrinkage by multiple fronts, is expected to favour the onset and persistence of anti-tumour immunity, enhancing T cell co-stimulation, DC functions, and counteracting tumour immune-evasions mechanisms. Obviously, this strategy still need scientific support from basic research into the mechanism(s) of action related to cell death pathways accounting for immune activation (Haynes, van der Most et al. 2008).

However, although the ability of some conventional agents to alert the immune system about the presence of a tumour may be exploited to provide a multiple-sided attack against cancer cells, the risk of selecting resistant tumour clones still remains. Indeed, genetic instability, which intrinsically characterizes cancer cells, can allow neoplastic cells to acquire survival and/or growth advantages, being thus positively selected. Stromal cells in the tumour microenvironment, being genetically stable and possessing limited proliferative capacity compared to cancer cells, may represent alternative valuable target for anticancer treatment, since the risk of their escape during therapy is exceedingly lower.

1.3 Aims of the thesis

The impaired tumour-specific immune activation in indolent NHL patients as well as their sensitivity to immunotherapeutic intervention are well documented (Dave, Wright et al. 2004; Anichini, Mortarini et al. 2006a). As a step forward to improve active immunotherapy approaches for the treatment of these diseases, we developed a fully autologous *ex-vivo* DC-vaccine using as antigenic cargo whole autologous tumour cells killed by heat shock, γ-irradiation and UV-C exposure. This strategy was expected to favour the expression of "danger signals" and the activation of DCs for the *in-vivo* priming of an adaptive immune response against a wide spectrum of TAAs. Eighteen patients with measurable indolent NHL who had relapsed after at least one chemo-radiotherapy regimen were enrolled in a pilot trial for the study of this vaccine. Vaccination achieved six objective clinical responses, including three continuous complete responses and three partial responses.

On the basis of these preliminary results, the aims of the thesis have been:

- To verify whether a positive clinical outcome after vaccination correlated with the induction of tumour-specific immune activation;
- To identify potential predictors of the clinical outcome of indolent B-NHL patients treated with a fully autologous DC-vaccine;
- To identify the NHL-associated/-specific antigens that evoked a humoral response associated with the clinical efficacy of vaccination, with the aim to exploit them as novel targets of passive immunotherapy;
- To study potential ways of intervention on the host immune system and/or the tumour microenvironment to increase the frequency of immunological and clinical responses to anticancer immunotherapy in indolent NHL patients.

2 IMMUNOLOGIC AND CLINICAL RESPONSES TO DC-BASED VACCINATION IN INDOLENT NHL PATIENTS

2.1 Introduction

Immunotherapy trials targeting the tumour B-cell Id have documented clinical responses in a sizeable number of patients and the promotion of B- and T-cell activation against the autologous tumours in the majority of vaccinated patients (Kwak, Campbell et al. 1992; Hsu, Caspar et al. 1997; Bendandi, Gocke et al. 1999; Timmerman 2002; Redfern, Guthrie et al. 2006). However, three phase-III clinical trials of Id vaccination have indicated the lack of clinical benefit in terms of progression/disease free survival (BiovaxId, Biovest International, Schuster S. J. ASCO 2009 abs 27, S15; MyVax, Genitope, Levy et al. 2009; FavId, Favrille, (Freedman, Neelapu et al. 2009)).

Since the induction of a specific immunity against a single tumour antigen may be hampered by the emergence of escape variants (Thurner, Haendle et al. 1999), the use of whole tumour cell preparations as source of antigens to load DCs may circumvent these limitations. Furthermore, it has the advantage of widening the spectrum of TAAs that can be recognized and targeted by the immune system (Maier, Tun-Kyi et al. 2003; Neelapu, Gause et al. 2007), even though at the expense of a potential risk of autoimmunity. In particular, the strategy to pulse DCs with killed cells was already reported to favour the efficient transfer of TAA to the MHC class I processing pathway of DCs (Albert, Sauter et al. 1998; Berard, Blanco et al. 2000; Hus, Rolinski et al. 2005), thereby enabling TAA cross-presentation and CTL activation. In addition, exploiting the cytotoxicity of stress stimuli, such as heat shock and irradiation, to obtain the apoptotic and necrotic tumour cell bodies for DC loading, could even increase the immunogenicity of the vaccine through the expression and/or the release of immunogenic molecule, such as HSPs (Feng 2001; Zitvogel, Apetoh et al. 2008).

2.2 Aims of the Chapter

The aim of the work described in this Chapter was to deeply characterize the immunological responses of indolent NHL patients enrolled in a pilot trial of DC-based vaccination and to analyse their association with the clinical outcome, monitored at the Medical Oncology Unit of the *Fondazione Istituti di Ricovero e Cura a Carattere Scientifico (IRCCS) Istituto Nazionale dei Tumori di Milano,* under the supervision of Dr Massimo Di Nicola. Changes in post- compared to pre-vaccination circulating and/or tumour-associated T-cell and NK cell populations as well as tumour-reacting Abs were correlated with the clinical changes observed in the patients.

The information obtained aimed to provide a valuable insight not only for identifying those patients who would benefit from such a treatment but also in the development of improved lymphoma vaccines. The results reported here also formed the basis for additional studies on the immune response to the vaccine.

2.3 Materials and Methods

2.3.1 Patient characteristics

Eighteen patients with FL (n=12) or lymphoplasmocytoid lymphoma (n=6), who were relapsed after their previous treatment, were accrued to this pilot study between January 2003 and May 2005. The clinical characteristics of the patients are summarized in Table 2.1. The median number of previous treatment regimens was two (range 1-5). The study included 4 patients treated with high dose sequential chemotherapy (HDS) supported by autologous HSCT. Eligibility criteria for entry into the study were as follows: confirmed diagnosis of indolent B-NHL (lymphoplasmocytoid or FL grades I-IIIa), relapse after previous treatment, age ≥ 18 years old and Eastern Cooperative Oncology Group performance status ≤ 2. Patients were required to be at least 180 days from their last treatment, to have measurable disease and adequate haematological, renal, and hepatic functions. Patients were ineligible if they had more than six prior treatment regimens, concurrent immunosuppressive therapy, a history of central nervous system lymphoma, serious coexistent active medical problems, a history of a prior malignancy (excluding non-melanoma carcinomas of the skin and in situ cervical carcinomas) unless the patient was in remission for ≥2 years, or were pregnant or breast feeding. Analysis of the disease response to DC vaccination was performed using Cheson criteria (Cheson, Pfistner et al. 2007). Seven out of 18 patients were evaluated for clonal rearrangement of Bcl-2/IgH genes to evaluate minimal residual disease (MRD). All 18 patients were assessed for a response based on a central radiology and/or clinical evaluation. The study protocol was approved by the Italian *Istituto Superiore di Sanità* and by the Institutional Scientific Review Committee. Written informed consent for vaccination procedure and for the investigational use of lymphocytes, serum samples and tumour specimens was obtained from each patient before enrolling in the study, in accordance with the Declaration of Helsinki.

Table 0.1 Patients' characteristics.

Patient group/ UPN no.	Age(y)/ Sex	NHL type and stage	Previous treatment, response/duration	Sites of disease at vaccination (cm*)	Clinical Response, TTP	Post-vaccination Therapy	Current status, survival after vaccination
Clinically responding patients							
12	65/M	LP, Stage IA	-6 CVP (CR/36 months)	Periaortic LN (1.8); BM and PB	CR	No additional tx	CR, +67 mos
13	72/F	FL grade I, Stage IIA	-6 CVP (PR/16 months); -4 Rituximab (CR/24 months)	Mediastinal, axillary and supraclavicular LN (2)	CR	No additional tx	CR, +64 mos
14	52/F	FL grade IIIa, Stage IVA	-6 R-MegaCEOP (PR/10 months); -HDS (CR/6 months)	Cervical, axillary, mediastinal, periaortic and parailiac LN (2.5)	CR	No additional tx	CR, +63 mos
1	49/F	LP, Stage IVA	-6 CVP (CR/48 months); -8 Rituximab (PR/72 months)	Cervical, axillary, mediastinal, periaortic, periiliac LN (2.5); BM	PR, 47 mos	No additional tx	PD, +81 mos
5	52/M	FL grade I, Stage IVA	-6 CVP (CR/24 months); -4 Rituximab (PR/15 months)	Cervical, periaortic and periiliac LN (2.5); BM	PR, 12 mos	HDS autoBMT	PR, +74 mos
6	45/M	FL grade II, Stage IVA	-6 R-CEOP (CR/12 months); -4 Rituximab (PR/8 months); -HDS (CR/12 months)	Cervical, axillary, mediastinal, periaortic and inguinal LN (2.5)	PR, 7 mos	DHAP	Died, 22 mos
Patients who experienced the disease stabilization							
2	60/M	LP, Stage IVA	-6 CVP (PR/36 months); -6 Rituximab (CR/48 months)	Cervical, axillary and mediastinal LN (3); spleen; BM and PB	SD	No additional tx	SD, +60 mos
4	51/M	FL grade II, Stage IA	-4 Rituximab (CR/24 months); -RT 30 Gy (CR/24 months)	Inguinal and periliac LN (2.5)	SD	No additional tx	CR, +78 mos
9	63/M	LP, Stage IVA	-8 Rituximab (PR/16 months); -4 CVP (PR/18 months); -4 R-CHOP (SD/32 months)	BM (massive) and PB	SD	No additional tx	SD, +69 mos
10	54/M	FL grade I, Stage IVA	-8 R-CVP (CR/36 months)	Cervical, axillary, mediastinal, periaortic and parailiac LN (5);	SD	No additional tx	SD, +68 mos
11	72/M	FL grade II, Stage IVB	-18 mo Leukeran (CR/84 months)	Cervical, axillary, mediastinal, periaortic and inguinal LN (4); BM	SD, 10 mos	R-CHOP	CR, +68 mos
16	55/F	FL grade I, Stage IVA	6 CVP (CR/24 months)	Axillary, cervical, mediastinal and periaortic LN (3); BM	SD	No additional tx	CR, +55 mos
17	65/M	LP, Stage IIIA	8 CVP (CR/98 months)	Cervical, axillary, mediastinal and periaortic LN (3); speen;	SD, 10 mos	HDS autoBMT	CR +52 mos
18	62/F	FL grade I, Stage IIIA	6 R-CVP (CR/36 months); 4 Rituximab (CR/26 months)	Axillary, periaortic and periiliac LN (4)	SD	No additional tx	SD, +52 mos
Patients who experienced the progression of the disease							
3	60/M	FL grade II, Stage IVA	-8 CHOP (PR/16 months) -6 Rituximab (PR/8 months)	Axillary, mediastinal, periaortic, inguinal LN (3); spleen	PD, 1 mo	HDS autoBMT	CR +78 mos
7	50/M	FL grade I, Stage IVA	-6 CHOP (PR/12 months); -4 Rituximab (PR/6 months)	Cervical, axillary, mediastinal, periaortic and parailiac LN (6);	PD, 1 mo	HDS autoBMT	CR, +69 mos
8	56/M	FL grade II, Stage IA	-3 CHOP-bleo/3 CVP (RC/16 months); -HDS (CR/24 months); -8 Rituximab (CR/6 months)	Periaortic, periiliac and inguinal LN (3.2)	PD, 1 mo	AlloBMT	CR, +68 mos
15	72/M	LP, Stage IA	-3 CHOP (PR/8 months); -Splenectomy + RT (PR/12 months); -12 months Leukeran (PR/7 months); -HDS (CR/12 months); -4 Rituximab (PD/n.a.)	Cervical, axillary, mediastinal, periaortic and parailiac LN (6.5); BM and PB	PD, 1 mo	DHAP	Died, 7 mos

UPN: Unique Progressive Number; LP: lymphoplasmacytic lymphoma; Cheson criteria (Cheson, Pfistner et al. 2007) have been used to define the response to vaccination: complete remission (CR), disappearance of all evidence of disease; partial response (PR), regression ≥50% of measurable disease and no new sites; stable disease (SD), failure to attain CR/PR or PD; progressive disease (PD), any new lesion or increase by ≥50% of previously involved sites from nadir; TTP: time to progression; CVP: cyclophosphamide, vincristine and prednisone; R-CVP: rituximab plus CVP; CHOP: cyclophosphamide, adryamicin, vincristine and prednisone; R-CHOP: rituximab plus CHOP; RT: Radiotherapy; CEOP: cyclophosphamide, epiadryamicin, vincristine and prednisone; R-CEOP: rituximab plus CEOP; HDS: high dose sequential chemotherapy; autoBMT: autologous bone marrow transplantation; alloBMT: allogeneic bone marrow transplantation; LN: lymph-nodes; BM: bone marrow; PB: peripheral blood; DHAP: desametasone, cis-platinum, aracytin; tx: therapy. *maximum diameter of the largest lesion.

2.3.2 Preparation and injection of vaccine

All procedures for the preparation of the vaccine were carried out under GMP conditions.

Tumour cells were isolated from malignant LNs and, in case of circulating tumour cells, from PB of indolent NHL patients. In 16 patients, LNs were processed by mechanical dissociation (BD™ Medimachine, BD Biosciences, Franklin Lakes, NJ) to yield single-cell suspensions. In the remaining 2 patients, circulating tumour cells were harvested by leukapheresis. CD19+ NHL cells were purified from PB and/ or LN by using a high-gradient immunomagnetic technique according to the manufacturer's instructions (CD19 microbeads, Miltenyi Biotech, Gladbach, Germany). Tumour cells were heat shocked and then induced to undergo apoptosis by exposure to γ-irradiation and UVC rays as previously described (Di Nicola, Napoli et al. 2003). Briefly, $120x10^6$ CD19+ cells were resuspended in 10% autologous human serum-supplemented Iscove's modified Dulbecco's medium (Cambrex, Milan, Italy) and incubated for 60 min in a water bath at 46°C to induce the expression of HSPs. The cells were irradiated (150 Gy) and finally exposed to a T-UV9 UVC Germicidal Lamp (Philips, Holland) calibrated to provide 0.5 J/m2/sec UVC, for 25 sec, at a lamp-to-cell culture distance of 39 cm.

Autologous circulating monocytes, selected by a high-gradient immunomagnetic technique (CliniMacs CD14 microbeads, Miltenyi Biotech), were cultured for five days with recombinant human (rh)GM-CSF (Leucomax, Novartis, Basel, Switzerland) and rhIL-4 (kindly provided by Shering-Plough, USA) as described (Sallusto and Lanzavecchia 1994). Then, immature DCs were co-cultured for 48 hours with heat-shocked, γ-irradiated, and UVC-irradiated neoplastic B cells at a 1:2 ratio. Further DC maturation was promoted by adding TNF-α (Knoll, Ludwigshafen, Germany) for the last 12 hours of culture. Tumour-loaded DCs were finally resuspended in 1 ml of 0.9% NaCl to be injected subcutaneously into patients. Four administrations of the vaccine in close vicinity to axillary and inguinal LNs were repeated at two-week intervals.

2.3.3 Flow cytometry analysis

Monocyte-derived DCs and tumour-loaded DCs were labelled with the following mouse anti-human mAbs: fluorescein isothiocyanate (FITC)-labelled anti-CD14, peridinin chlorophyll protein (PerCP)-labelled anti-HLA-DR, FITC-labelled anti-CD19 (BD Biosciences, San Jose, CA), phycoerythrin (PE)-labelled anti-CD80, PE-labelled anti-CD83, PE-labelled anti-CD86 (BD Pharmingen), PE-labelled anti-CD1a (Coulter, Krefeld, Germany). Tumour loaded-DCs were co-stained with propidium iodide (PI, Bender Medsystem, Wien, Austria) and anti-CD19 mAb to verify that unloaded tumour B cells present in the culture were nonviable.

The maturation status of T cells at tumour site was assessed by labelling LN cell suspensions with the following mouse anti-human mAbs: PE-labelled anti-CD45RA (BD Pharmingen), FITC-labelled anti-CD4, PerCP-labelled anti-CD8 and allophycocyanin (APC) - labelled anti-CCR7 (BD Biosciences, San Jose, CA).

NK cells were characterized by staining PB samples with FITC-labelled anti-CD56 (BD Biosciences), PE-labelled anti-CD16 (Miltenyi Biotech), PerCP-labelled anti-CD3 (BD Biosciences) and APC-labelled anti-NKG2D or anti-NKp46 mAbs (BD Pharmingen).

The ability of PB myeloid DCs to acquire co-stimulatory properties *in vivo* were studied by staining PBMCs with mouse anti-human FITC-labelled OX40L (Ansell, Bayport, MN), PE-labelled anti-CD1c or anti-CD14, PerCP-labelled anti-CD45, and APC-labelled OX40 (eBioscience, San Diego, CA) mAbs.

The percentage of Tregs was evaluated by intracellular FOXP3 staining of PB and LN cell samples collected before and after vaccination, using the "FITC-labelled anti-human FOXP3 staining kit" (eBioscience) according to the manufacturer's instructions, after staining cells with PE-labelled anti-CD25 (Miltenyi Biotech), PerCP-labelled anti-CD3, and APC-labelled anti-CD4 (BD Bioscences) mAbs.

Samples were acquired by setting photomultiplier tubes on the autofluorescence of the related negative control. All plots were gated on high Forward Scatter (FSC) and low Side Scatter (SSC) to exclude cell debris. A minimum of 50.000-gated events was collected per sample. Data were acquired on a BD fluorescence-activated cell sorting (FACS)Calibur using BD CellQuest software version 3.3 (Becton Dickinson, Lincoln Park, NJ) and analyszed by FlowJo 8.7.1 software version for Macintosh (Tree Star, Inc. Ashland, OR).

2.3.4 Amplification and sequencing of tumour-specific IgH and Bcl2/IgH rearrangements

Molecular monitoring of the MRD (7/18 patients) and sequencing of Ig genes expressed by neoplastic B cells (patient #14) were performed at the *Molecular Diagnostic Unit* of our Institute. Either nested PCR amplification of the Bcl-2/IgH or semi-nested PCR amplification of the clonal rearrangement of IgH genes when Bcl-2 was not amplified were used (Corradini, Astolfi et al. 1997). In the latter instances, DNA obtained from paraffin-embedded sections of diagnostic biopsies from each patient was PCR-amplified using VH and JH consensus primers for the framework region 2 of the IgH gene. In order to obtain a patient-specific primer, the DNA was sub-cloned into a suitable plasmid and the PCR products were sequenced. DNA extraction from BM and PB samples was performed using the QIAamp DNA blood extraction kit (Qiagen, Valencia, CA, U.S.A.). A patient-specific positive control was included in all PCR experiments. Negative controls contained water instead of DNA. All PCR reactions were performed in duplicate for a minimum of two independent experiments.

2.3.5 Peptide prediction and HLA stabilization assay

The identification and the production of HLA-A*0201-binding peptides in the HLA-A*0201+ patient (#14) was performed by PRIMM s.r.l (San Raffaele Biomedical Science Park, Milan, Italy). Briefly, potential binding peptides of 9-10 amino acids were selected using three different algorithms (http://bimas.dcrt.nih.gov/molbio/hla_bind; http://www.syfpeithi.de/; and http//hlaligand.ouhsc.edu/SBT.htm) within the tumour-specific IgH sequence from the HLA-A*0201+ patient (#14). Three peptides, showing high scores by all three algorithms were selected: peptide 1 (sequence: TLLRLLFNWV) at the boundary between complementarity determining region (CDR) 1 and framework region (FR) 2; peptide 2 (QLVQSGAEVK) from FR1, and peptide 3 (YCARVPAGV) from CDR3. Synthetic peptides corresponding to these sequences were then screened for binding to HLA-A2 by the HLA stabilization assay using TAP-deficient T2 cells, as described (Sensi, Nicolini et al. 2005), using the HLA-A2-specific Abs CR11.351 and BB7.2 (ATCC, Manassas, VA). HLA-A2 stabilization was expressed as the Fluorescence Index (F.I.) (Trojan, Schultze et al. 2000) based on the mean fluorescence intensity (MFI) for HLA-A2, as follows: [MFI(T2 loaded with peptide)/MFI(empty T2)]-1.

2.3.6 ELISPOT assays

The IFN-γ and the IL-4 ELISPOT assays were performed as described with minor modifications (Di Nicola, Carlo-Stella et al. 2004). Briefly, plates were coated at 4°C overnight with 10 μg/ml of anti-IFN-γ mAb (clone 1-D1K) or 10 μg/ml of anti-IL-4 mAb (clone IL-4-I) (Mabtech, Stockholm, Sweden). PB-derived lymphocytes were stimulated with the synthetic peptides 1, 2 or 3 (30 μg/ml). Treatment with 1% phytohemagglutinin (PHA) (for IFN-γ ELISPOT) or phorbol 12-myristate 13-acetate (PMA) plus ionomycin (for IL-4 ELISPOT) was used as positive controls. After incubation at 37°C for 48-72 hours, plates were labelled for 2 hours at room temperature (RT) with 1 μg/ml of biotin-conjugated secondary anti-human IFN-γ (clone 7B6-1) or IL-4 (clone IL-4-II) mAbs (Mabtech). After the addition of alkaline phosphatase (ALP)-conjugated streptavidin (Mabtech), the plates were developed with an ALP-conjugate substrate kit (Bio-Rad Laboratories, Hercules, CA). The number of spots for each well was evaluated using the AID ELISPOT reader (Autoimmun Diagnostika GmbH, Strasburg, Germany). The number of spots produced by unstimulated PB lymphocytes was <20% of the number of spots obtained in response to peptides and was subtracted from the latter values.

In some experiments, CD3+ T cells were purified by immunomagnetic sorting (Pan T cell isolation kit, Miltenyi) from involved LNs that were surgically removed before and after vaccination from three patients that achieved a partial response. These cells were then cultured for one week in the presence of autologous neoplastic B cells in medium supplemented with IL-2, and then evaluated by IFN-γ-ELISPOT in response to autologous or allogeneic tumour B-cell challenge.

2.3.7 Purification of human Ig, biotinylation, and immunohistochemistry

Ab purification from human sera was carried out using the MultiClear kit (Cabru, Lesmo, Milano, Italy) following the manufacturer's instruction. After purification, total human Igs were biotinylated as described (Bayer and Wilchek 1990). Biotinylated Abs were used for immunohistochemistry (IHC) analyses on formalin-fixed, paraffin-embedded tissue using heat-induced antigen retrieval with 10 mmol/L of citrate buffer (0.07M pH 6.0, Bioptica, Milan, Italy), as described with minor modifications (Anichini, Mortarini et al. 2006b). Briefly, sections were incubated with biotinylated patients' Abs for 1 hour at RT, washed, and then incubated with peroxidase-conjugated streptavidin. Immune complexes were visualized by 3,3'-diaminobenzidine (Sigma FastTM Sigma Chemical Co.) and hematoxylin counterstaining.

2.3.8 Statistical analysis

Analyses of statistical significance were performed using the two-sided Student's *t* test. When the sample size was small, the Fisher exact test or the non-parametric Mann-Whitney test was used. Statistical analyses were performed on the Prism 5.0a software version for Macintosh (GraphPad Software, Inc.). The significance of the results was annotated as follows: *: $p \leq 0.05$;**: $p \leq 0.01$; *** : $p \leq 0.001$.

2.4 Results

2.4.1 Quality control of vaccine preparations

The preparation of the vaccine was carried out when the percentage of the malignant B-cell clone (IgG κ or λ) was ≥ 75%. The mean number of CD19 positively selected tumour cells was 430 ± 35 x10^6, with ≥ 90% cell viability, as determined by the Trypan blue exclusion test. PB-derived monocytes were cultured and skewed toward CD14-CD1a+CD83- immature DCs in the presence of rhGM-CSF and rhIL-4, as described (Sallusto and Lanzavecchia 1994). After five days of culture, the average number of DCs was 250 ± 50 x10^6, with ≥ 90% cell viability, as determined by the Trypan blue exclusion test. After co-culture with killed and heat shocked tumour cells, the immature DCs differentiated towards a mature phenotype (CD80+, CD86+, CD83+, HLA-DR+, CD1a+) (data not shown). At the end of co-culture, vaccine preparations did not contain live CD19+ tumour cells, since B cells that eventually appeared in the cultures stained positive for propidium iodide. The mean number of injected DCs was 45 ± 3x10^6. No microbiological contaminations were observed in any stages of the vaccine preparation.

2.4.2 Safety and clinical efficacy

All of the 18 enrolled patients received 4 doses of autologous vaccine. The only observed adverse events were injection site reactions that occurred in 66% of treated patients. Injections site reactions were transient and none of the patients required medication. In particular, no significant reduction of B cells or Ig levels in the circulation was observed during or after injections (data not shown). No signs of autoimmunity were detected, as measured by the absence of C-reactive protein and anti-nuclear antibodies (data not shown).

All patients were monitored for immune responses to the vaccine with a median follow-up of 68 months (range 7-81 months). Objective responses were identified in 6 out of 18 patients (33.3%) (Table 2.1), these included 3 complete and 3 partial responses and are summarized in Table 2.1. Complete responder patients (CR) received the vaccine whilst in relapse after the last chemotherapy regimen (n. 12, 13, 14, Table 2.1). Their overall tumour mass gradually reduced after starting the vaccination programme and completely disappeared after a median of 6 months from the last injection, when clinical and molecular responses were attained (Figure 2.1A for a representative example). Noteworthy, all CR have experienced a longer-lasting remission compared to the previous one obtained with standard regimens.

Partial responders (PR) achieved the response after a median of 5 months following the last vaccine injection, which lasted for a median of 12 months (n. 1, 5, 6, Table 2.1). Unfortunately, in this case, the vaccine was not able to induce a longer response compared to that achieved after the last therapy prior to enrolment in the present study, and the patients experienced tumour progression (Figure 2.1B for a representative example).
All non-responding patients (NR) had conspicuous tumour burden or bulky disease at the time of enrolment. Disease stabilization was observed in 8 patients with a median duration of 61.5 months. Interestingly, in 2 patients, tumour underwent progressive shrinkage until the achievement of a complete remission after two years from the last vaccine injection (n. 4 and 16, Table 2.1). The last 4 patients progressed 1 month after the last vaccination.

Three out of 4 patients are alive after salvage high-dose sequencial chemotherapy supported by auto or allograft. One patient died following disease progression.

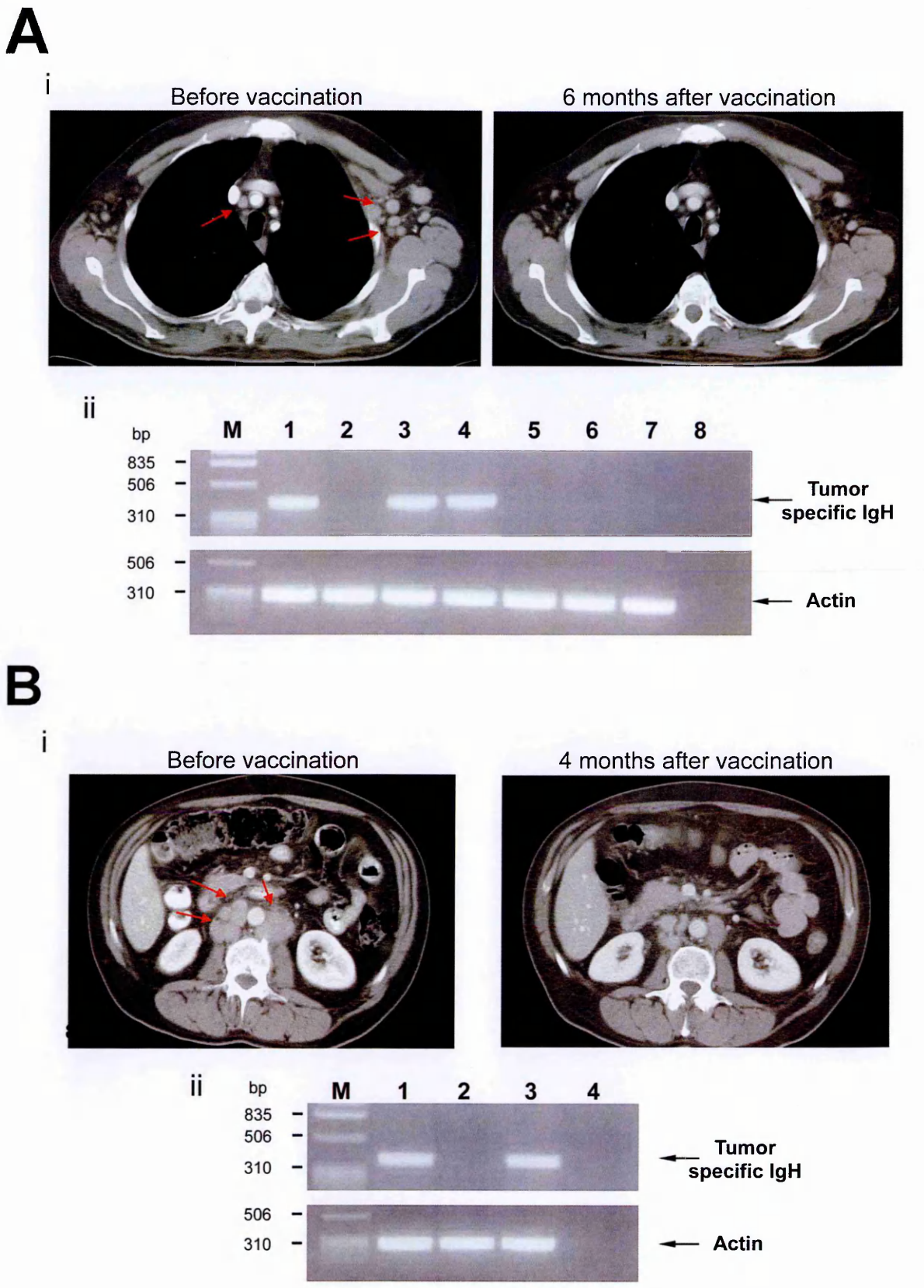


Figure 0.1 Therapeutic effects of vaccination.

(A) Example of a clinical CR. CT scan images of the chest of patient #12 revealed multiple small enlarged mediastinal and left axillary LNs (i, left, red arrows) that normalized six months after vaccination (i, right). (ii) Involvement of BM (lane 1 and 2, before vaccination and 6 month after vaccination, respectively) and PB (lane 3-7, before vaccination, 3, 6, 12 and 18 months after vaccination, respectively) was evaluated by PCR amplification of the clonal tumour-specific IgH gene rearrangement using IgH specific molecular probe. Actin amplification was performed as a control. Line 8, no DNA. M, marker. (B) Example of a clinical PR. CT scan images of the retro-peritoneum of patient #5 showed enlarged retroperitoneal LNs (i, left, red arrows) that shrunk four months after vaccination (i, right). (ii) Involvement of BM (lane 1-3, before vaccination, 4 and 12 months after vaccination, respectively) was evaluated by PCR amplification for panel A. Actin amplification was performed as a control. Line 4, no DNA. M, marker.

2.4.3 Biological efficacy

2.4.3.1 Tregs

Recent findings demonstrated that the expansion and induction of suppressor cells with a "naturally occurring" CD25+CD4+FOXP3+ Treg phenotype in PB and in the tumour microenvironment of B-NHL patients is a crucial factor contributing to the tumour immune evasion (Mittal, Marshall et al. 2008). To verify whether DC-based vaccination could counteract this mechanism and restore the physiologic balance of Tregs in PB and/or tumour site, the frequency of CD4+CD25+FOXP3+ T cells was evaluated in enrolled patients before and after vaccination. At the time of the enrolment, NHL patients showed a higher frequency of Tregs in PB (p= 0.0096) and LNs (p<0.0001) in comparison to healthy donors (Figure 2.2A). Interestingly, PB Treg frequency significantly decreased within 6 months following vaccination, but only in R (p= 0.0076; Figure 2.2A-Bi-ii). Treg absolute counts confirmed these data (data not shown). In addition, CD4+CD25+FOXP3+ Treg frequency at tumour sites was considerably reduced at the time of response in PR (Figure 2.2Biii). Accordingly, when the disease progressed, the percentage of Tregs increased again to pre-therapy values at tumour site (Figure 2.2Biii).

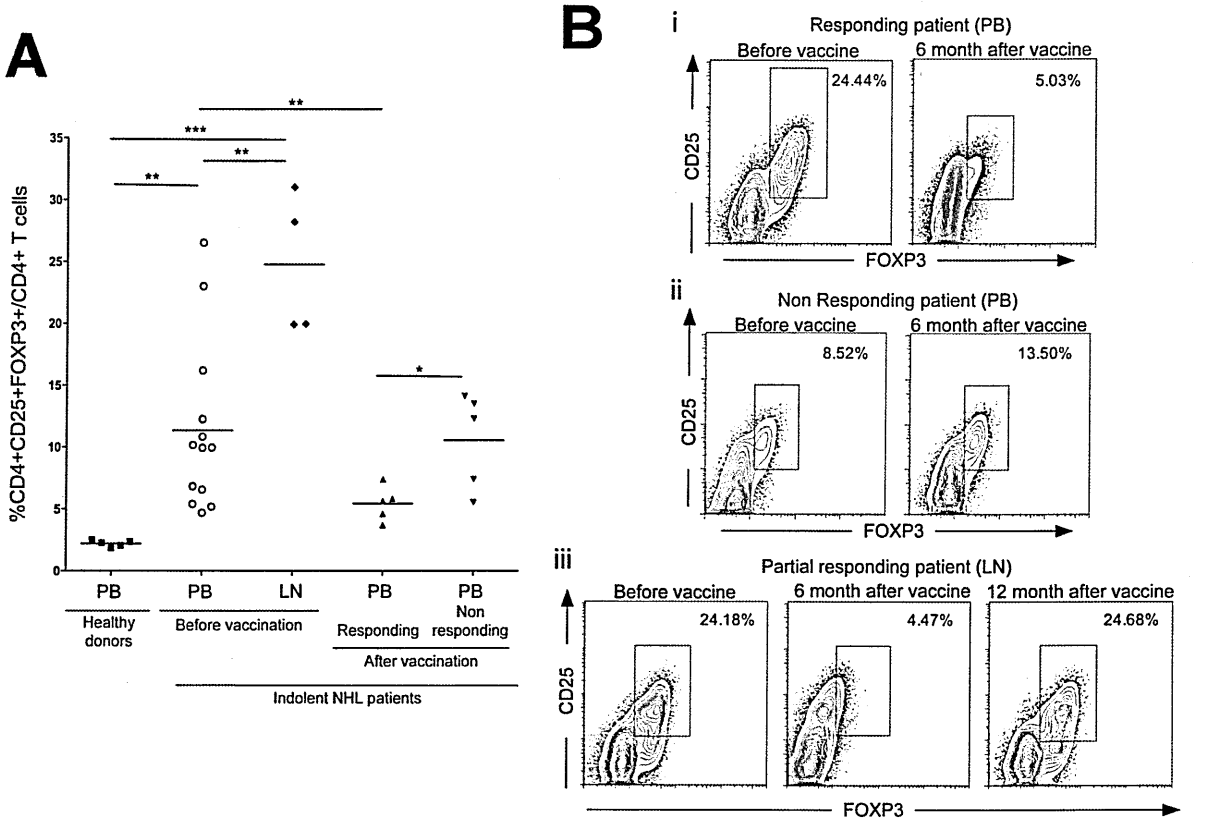


Figure 0.2 Evaluation of Treg frequency in PB and LN of NHL patients before and after vaccination. (A) Schematic comparison of CD4+CD25+FOXP3+ T cells frequencies in donors' or patients' PB and LNs before vaccination and in Rs' and NRs' PB after vaccination. Statistically significant differences, by two-sided Student's *t* test, are reported (*: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$). (B) Representative examples of Treg frequencies in pre- versus post-vaccine PB of complete R #14 (i), NR #7 (ii), and in LN specimens collected before vaccination, after 6 (at the time of partial remission) and 12 months (at disease progression) from the last vaccine administration in PR #5 (iii). All plots were gated on CD3+CD4+ cells.

2.4.3.2 NK cell activity

As a possible explanation for the observed Treg reduction in Rs, the frequency and phenotype of circulating NK cells were analysed before and after vaccination. This was because upon activation NK cells would be expected to prevent Treg differentiation, promote T cell proliferation and effector functions (Zingoni, Sornasse et al. 2004; Brillard, Pallandre et al. 2007). By studying the ratio of post- versus pre-vaccination cytotoxic NK cell (CD3-CD56dimCD16+) frequencies in PB samples, it was found that after vaccination the NK cells of Rs expressed CD16 at a higher frequency (p=0.009; Figure 2.3A) and intensity (p=0.0084; Figure 2.3B) compared to NRs (Figure 2.3C).

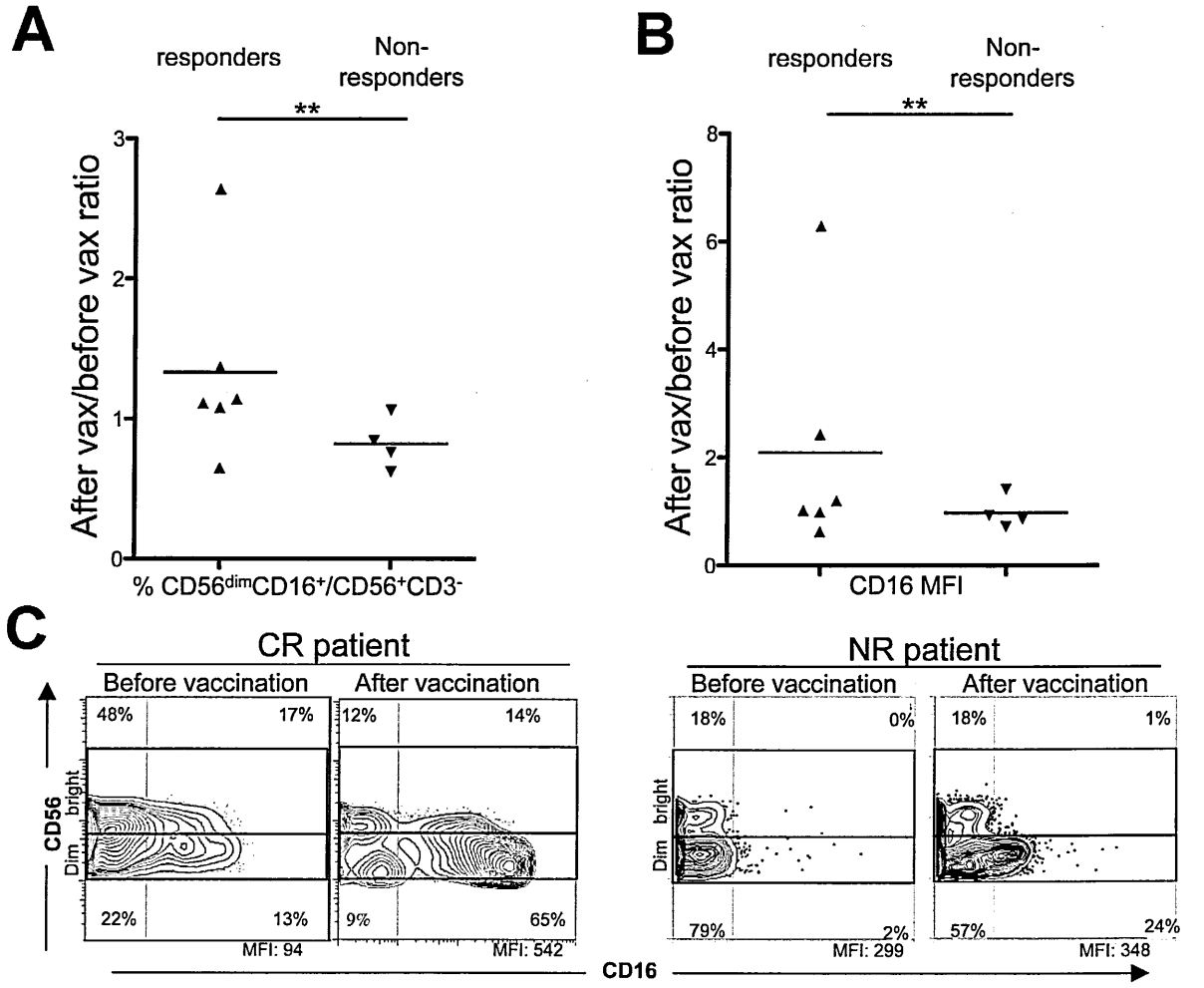


Figure 0.3 Evaluation of CD56dimCD16+ NK-cell frequency in NHL patient PB before and after vaccination.

(A) Ratios of CD56dimCD16+ NK frequencies in post- versus pre-vaccination PB samples of R and NR. (B) Ratios of post- versus pre-vaccination circulating NK cell CD16 MFI in R and NR. Statistically significant differences were calculated using the Fisher exact test (**: $p \leq 0.01$). (C) Representative examples of CD56dimCD16+ NK cell frequencies in pre- versus post-vaccine PB of a complete R (left panels, patient #14), and of a NR (right panels, patient #7). All plots were gated on CD3-CD56+ cells.

Moreover, NK cells from 4 out of 5 Rs analysed showed increased expression of the NKp46 activating NK cell receptor after vaccination. In contrast, NK cells from just 1 out of 4 NRs displayed a similar fold change in NKp46 expression (p=0.0442; Figure 2.4).

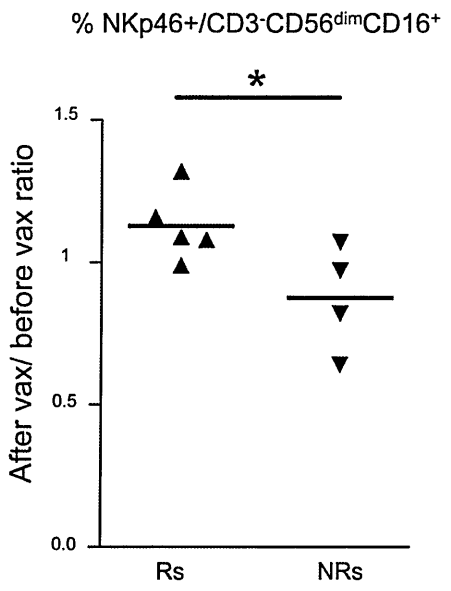


Figure 0.4 NKp46+ NK-cell frequency in NHL patient PB before and after vaccination.
Ratios of post- versus pre-vaccination NKp46+CD56dimCD16+CD3− NK cell frequencies in R and NR PB. Statistically significant differences were calculated using the two-sided Student's *t* test (*: $p \leq 0.05$).

2.4.3.3 Immunophenotype of conventional CD4 and CD8 T-cell populations

The function and maturation phenotype of T cells from vaccinated patients were also studied. T cells producing IFN-γ in response to autologous tumour challenge were found in pre-vaccine LNs and at higher frequency compared to that of T cells responding to an allogeneic HLA-mismatched B-cell tumour challenge, included as control (Figure 2.5A). The frequency of IFN-γ-producing T cells stimulated by the autologous B cell tumour was significantly increased in LNs harvested 6 months after the last vaccine administration in PR (Figure 2.5A, patient #1 and 5). A similar assay carried out in NRs after vaccination did not provide evidence for an increase of IFN-γ releasing T cells against the autologous tumour (Figure 2.5A, patient #10). Comparison of pre- versus post-vaccine maturation phenotype of tumour infiltrating CD4+ and CD8+ T cells from 2 of 3 PRs showed a shift towards the more differentiated CCR7-CD45RA- T_{EM} and/or CCR7-CD45RA+ T_{TD} phenotypes after vaccination (Figure 2.5B, patients #1 and 5). The maturation of TILs cells was not modified in NRs after vaccination (Figure 2.5B, patient #10).

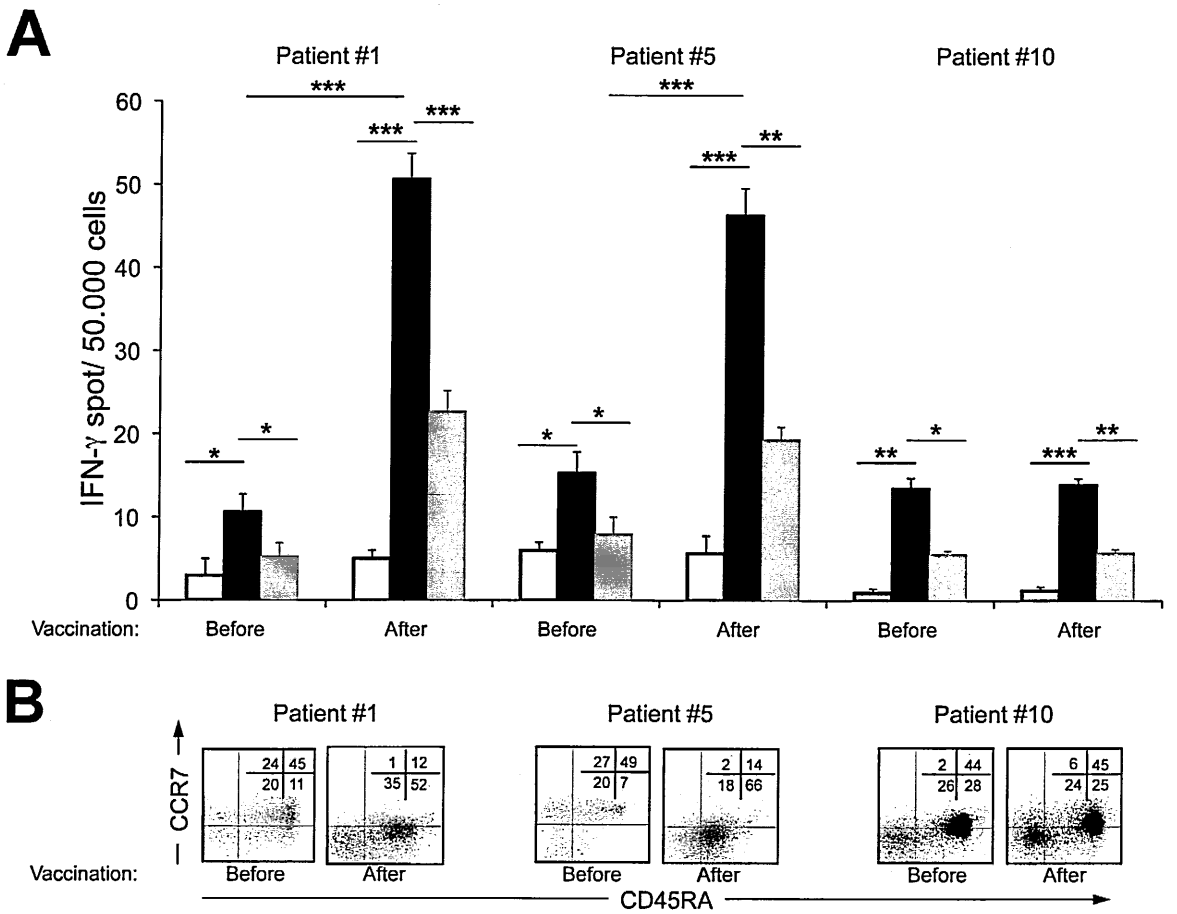


Figure 0.5 Functional and phenotypic analysis of T cells at tumour site from PRs or NRs.
T cells, isolated from malignant LNs before and after vaccination, were evaluated by IFN-γ–ELISPOT assay when cultured alone (white), with the autologous tumour (black) or an allogeneic tumour (grey). Results obtained with 2 of the 3 partial R and with one NR are reported. Error bars indicate standard deviation of the mean. Statistically significant differences, by two-sided Student's *t* test, are reported (*: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$). (B) Maturation profile of T cells at tumour site before and after vaccination was assessed by flow cytometry analysis of CCR7 and CD45RA expression in CD3+CD8+ gated cells.

In contrast to the results obtained with TILs from PR, IFN-γ ELISPOT with PB lymphocytes from both Rs and NRs failed to show evidence of an increase in T cells reacting against autologous tumour cells after vaccination (data not shown). However, vaccination seemed to provide some activation of the immune system even at the PB level, since the expression of co-stimulatory molecules, such as OX40L, increased in both CD141+ and CD1c+ circulating myeloid DCs of treated patients that achieved at least the stabilization of the disease (data not shown). In addition, the potential occurrence of a T-cell response against the tumour Ig-Id was evaluated in one CR that expressed the HLA-A*0201 antigen. Indeed, this provided the opportunity to evaluate the frequency of T cells specific for HLA-A2-binding peptides (Figure 2.6B) derived from the tumour-specific IgH sequence in pre- and post-vaccination PB samples (Figure 2.6A). Using the HLA stabilization assay, all 3 selected peptides were found to bind to HLA-A2 molecules as indicated by F.I. values after staining T2 cells with two different anti-HLA-A2 mAbs (Figure 2.6B). By ELISPOT assay, an increased frequency of T cells releasing IL-4 or IFN-γ (Figure 2.6C) in response to all the three peptides was found in post-vaccine PB samples of this patient in comparison to pre-vaccine samples ($p<0.05$). Amongst the three tested peptides, the CDR1- and FR1-encoded peptides gave rise to the strongest and longest-lasting response, which was detectable up to 517 days (for IFN-γ; Figure 2.6, right) or 291 days (IL-4; Figure 2.6, left) after the first vaccine administration. However, 291 days after

vaccination, a significant increase in the frequency of circulating T cells releasing IL-4 was also found in response to the CDR3-encoded peptide stimulation (Figure 2.6C, left).

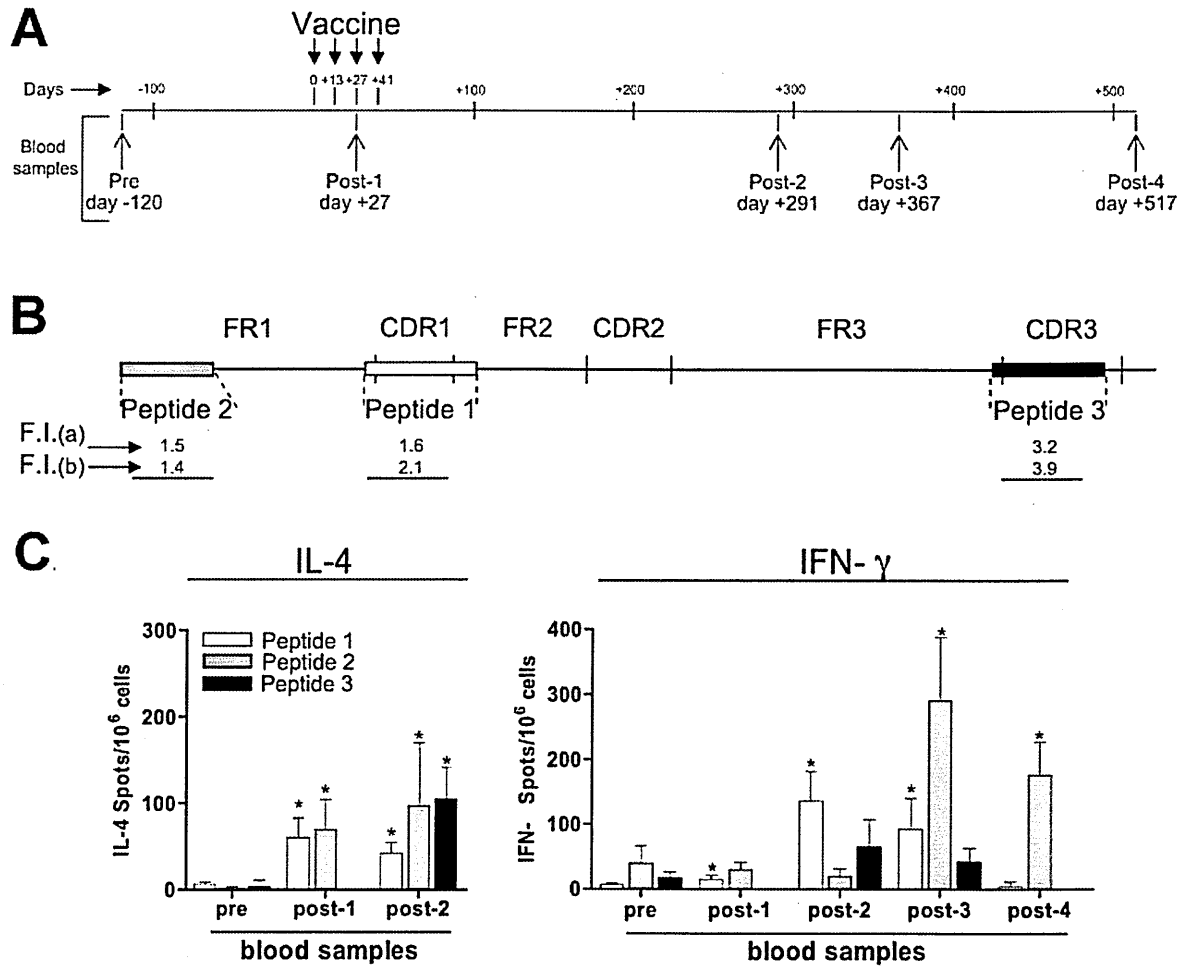


Figure 0.6 Circulating T cells directed against tumour-specific IgH-encoded epitopes in one CR. (A) PB samples were obtained from patient #14 at the indicated time points before (pre) and after (post) vaccination. (B) Synthetic peptides (indicated as peptides #1, #2 and #3) corresponding to CDR1, FR1 and CDR3 sequences of the tumour-specific IgH gene from patient #14 were used to assess cytokine release by circulating T cells. F.I. Fluorescence Index, as evaluated by the HLA-A2 stabilization assay after staining T2 cells with mAb CR11.351 (a) or BB7.2 (b). (C) Frequencies of IL-4 or IFN-γ-producing T cells against peptides #1, #2 and #3, as evaluated by ELISPOT in blood samples taken at the indicated time points before and after vaccination from patient #14. The HLA-A*0201-binding HIV peptide ILKEPVHGV was used as negative control in both the IL-4 and IFN-γ assays. Response to this peptide was <10 spots/10^6 cells in all blood samples analysed. *: Frequency of cytokine-releasing T cells, in the indicated blood samples, was significantly higher compared to pre-vaccine values (Mann-Whitney test, $p<0.05$).

2.4.3.4 AutoAb response

Initially, to determine whether Rs also developed a tumour-specific Ab response, Igs were purified from pre- and post-vaccine serum samples of two Rs and two NRs, and used to immunostain autologous tumour biopsies (Figure 2.7, for representative examples). An increased tumour-restricted reactivity was found only when post-vaccine Igs from R were compared to the matched pre-vaccine samples (Figure 2.7A-B and 2.7C-D). Normal cells in the tumour biopsies from these patients showed no immunostaining (Figure 2.7A-B and E, arrows). No tumour-restricted immunostaining was detected with pre- and post-vaccine biotinylated Igs of the two NR (Figure 2.7E-F, for a represenatative example). These results

may suggest that vaccination promoted also the induction of a tumour-specific B-cell response in R.

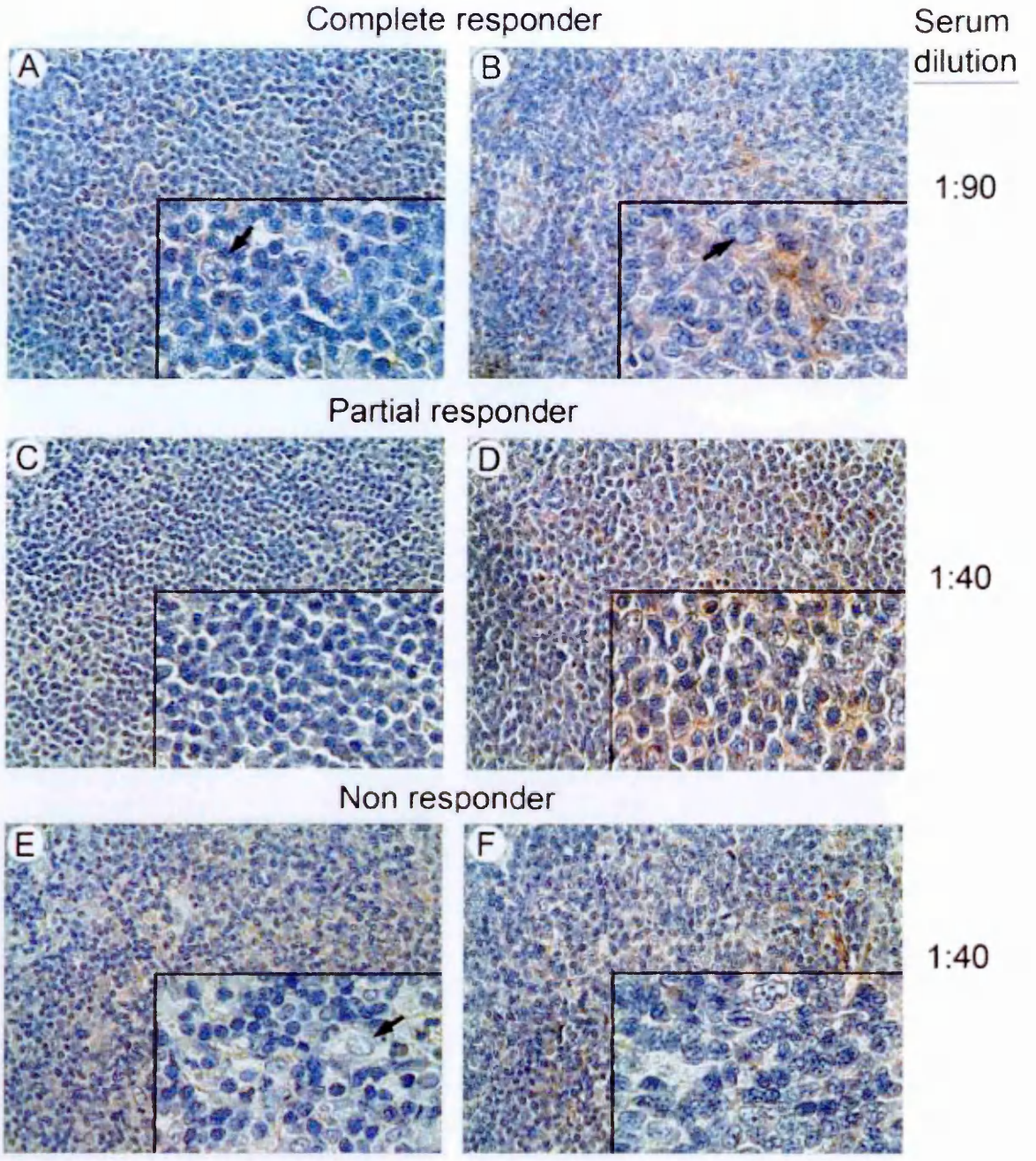


Figure 0.7 Immunohistochemical analysis of tumour biopsies with purified Igs of vaccinated patients. IHC analysis using biotin-conjugated Igs purified from pre- (A, C and E) and post-vaccine (B, D and F) serum samples on autologous NHL specimens of one CR (A-B), one PR (C-D) and one NR (A-F). Biotin-conjugated Ig samples were used at the indicated dilutions. Arrows indicate non-neoplastic follicular DCs in the inset of panels A, B and E. Panel insets are shown at x40 original magnification, panels at x10 original magnification.

2.5 Discussion

This pilot study tested the efficacy and the activity of a vaccination programme using autologous DCs loaded *ex-vivo* with autologous whole dying tumour cells for the treatment of indolent NHL patients with measurable disease. Vaccination resulted clinically efficacious in 6 out of 18 patients, achieving 3 objective radiographic complete and 3 partial responses. In all 3 CR, the duration of the clinical response after vaccination was longer compared to the duration of the first and second complete remissions obtained with the previous treatments. In agreement with a recent study of a patient-specific Id vaccination (Inoges S, Rodriguez-Calvillo M, et al. 2006), second longer remissions indicate a therapeutic effect. Differently from the study of Bendandi and colleagues (Bendandi 1999; Bendandi 2006), the clinical results obtained in this study were observed in patients that were not in chemotherapy-induced remission at the time of vaccination.

In several published trials in indolent NHL patients, immunological and clinical responses were observed after a vaccination against the tumour specific Id used alone or with DCs as a natural adjuvant (Hsu Blood 1997; Timmerman 2002; Inoges, Rodriguez-Calvillo 2006). Unexpectedly, 3 randomized phase-III studies, which started to prove the clinical benefit of Id-vaccination, failed to demonstrate an improvement in the progression/disease free survival of treated patients (Schuster et al. 2009; Levy 2009; Freedman, Neelapu et al. 2009). As pointed out by Bendandi (Bendandi 2006), the reasons of these failures may be related to the clinical features of FL disease and to the heterogeneous characteristics of patient-tailored Id-based immunotherapy. Nevertheless, in the Levy's study (Levy at al. 2009) the patients mounting Id-specific immune responses after vaccination showed a significant clinical benefit in term of time to progression. These results paved the way for identifying novel vaccination methods able to increase the probability to provide an anti-tumour immune activation associated with clinical benefit. For this purpose, in the present study, whole tumour cells were used as a source of antigen with the intent to elicit immune responses not only to tumour-specific IgH-encoded epitopes, but also to other potentially immunogenic known and unknown lymphoma-associated antigens (Brossart, Schneider et al. 2001; Bae, Martinson et al. 2005; Mikaelsson, Danesh-Manesh et al. 2005). In agreement with this hypothesis, Neelapu and colleagues (Neelapu, Gause et al. 2007) recently reported the clinical and immunological efficacy achieved in FL patients by a novel autologous whole tumour-derived proteoliposome vaccine formulation in a pilot clinical trial. Similarly, in this study, the antigenic cargo was represented by the whole proteic content of neoplastic B cells, but it was obtained by killing them with γ-irradiation and UVC rays after being heat-shocked in order to increase their immunogenicity (Zitvogel, Apetoh et al. 2008). The vast majority of studies of active immunotherapy targeting the lymphoma-Id have been designed to verify the role of vaccination in avoiding disease recurrences after chemotherapy with a de-bulking intent (Bendandi, Gocke et al. 1999; Timmerman 2002). The rationale of this strategy derived from the multiple observations that, in the presence of limited tumour burden, a vaccine-induced anti-tumour immune response could be more likely clinically effective. In the present study, instead, treated patients still presented clinically measurable disease. However, all the clinical responses were observed in patients with the smallest tumour burden, whereas, they were independent of the histological type, pre-vaccination chemotherapy (comprising HDS therapy), source of neoplastic cells for vaccine preparation (LN versus PB), DC maturation/activation profile and other clinical characteristics.

Immunological monitoring of the enrolled patients showed significant cellular and humoral immune activation in R as compared to NR. This indicates that the clinical responses observed after DC-based vaccination may be the result of a positive modulation of different components of the immune system. In particular, the reduction of Treg frequency after vaccination in both PB and LN of R might have favoured the occurrence of

anti-tumour responses by down-modulating Treg-mediated cancer tolerance (Zou 2006). Accordingly, tumour progression was found to be associated with the recurrence of increased Tregs in both in PB and involved LNs.

Although the mechanisms accounting for the observed down-modulation of Tregs in Rs after vaccination remain to be fully elucidated, recent findings reported that NHL cells can recruit Tregs by production of chemokine CCL22 (Yang, Novak et al. 2006). In addition, conventional T cells can be converted to functionally competent Treg within the tumour microenvironment (Colombo and Piconese 2007)(Mittal, Marshall et al. 2008). In support of the latter hypothesis, it has been shown that B-NHLs may produce regulatory factors, such as TGF-β, which in turn could promote the generation/expansion of Tregs. Tumour shrinkage in R after vaccination may thus partially explain the rebalance of Treg frequency (Chen, Jin et al. 2003; Huber, Schramm et al. 2004). Treg reduction may also result as a direct consequence of a successful immunisation. In agreement with this mechanism, the reduction of circulating Tregs was also recently described in breast cancer patients immunized with a HER-2 neu peptide vaccine that showed a concurrent anti-tumour CTL response (Hueman, Stojadinovic et al. 2006). Lastly, the activation of NK cells in R after vaccination might have also contributed to Tregs down-modulation (Brillard et al. 2007) (Roy, Barnes et al. 2008). These cells indeed can inhibit peripheral Treg conversion and lyse Tregs directly in a NKG2D- and NKp46-dependent manner (Brillard et al. 2007; Roy, Barnes et al. 2008). Accordingly, in R, in whom the parallel activation of NK and reduction of Tregs occurred, NK cells expressed higher levels of the activating receptor NKp46 after vaccination. Recent findings have yielded promising insights regarding the involvement of NK cells in the establishment of adaptive immunity in response to immunisation with DC-based vaccines (Osada, Clay et al. 2006; Woo, Clay et al. 2006). The interaction between mature DCs and NK cells was shown to stimulate NK cell proliferation, cytolytic activity and IFN-γ production (Bigley, Spence et al. 2010). In turn, activated NK cells can provide key signals for DC maturation, favouring the onset of an adaptive immune response against tumours that usually preclude the effective DC maturation through different immune escape mechanisms (Bigley, Spence et al. 2010). Accordingly, following vaccination, circulating myeloid DCs expressed higher levels of the co-stimulatory molecule OX40L. The immune modulation observed in Rs may thus reflect precise coordination of different immunological arms by *ex-vivo* matured DCs to properly present tumour antigens *in vivo*. In fact, as shown by Martin-Fontecha and colleagues (Martin-Fontecha, Thomsen et al. 2004), the injection of mature DCs can promote the activation of NK cells that, in turn provides a source of IFN-γ that is needed for a Th1 polarization. Therefore, it is possible that, activating NK cells, DC-based vaccines may lead to the reduction of FOXP3+CD25+CD4+ Tregs, thus favouring the onset of a tumour-specific immune response associated with clinical benefit.

The frequency of anti-tumour T cells was significantly higher after vaccination at the tumour site in PRs but not in NRs, suggesting that immune modulation promoted by the vaccine contributed to the observed specific immune responses. In addition, in these Rs, the immunological intervention induced the differentiation of CD4+ and CD8+ TILs towards an activation phenotype, consistent with the promotion of T cell effector functions at tumour sites (Oehen and Brduscha-Riem 1998). Nevertheless, the IFN-γ ELISPOT detected no increase of circulating tumour-specific T-cell frequency in Rs after vaccination. It is likely that in PB they were below the assay detection limit, probably due to their selective homing to tumour sites. Accordingly, when ELISPOT assays were carried out against tumour-specific IgH peptides instead of whole tumour cells, thus increasing the probability of specific T cells to become activated, post-vaccine PB frequency of IFN-γ or IL-4-releasing lymphocytes against IgH-encoding HLA-A*0201 peptides resulted higher to that observed in pre-vaccine samples of the same R. The responses against these epitopes were observed up to 17 (for IFN-γ) and 10 (for IL-4) months after vaccination, suggesting

that immunisation with tumour-loaded DCs has long lasting effects on the immune response to tumour-specific IgH-encoded peptides, as reported with Id-specific vaccination (Timmerman, Czerwinski et al. 2002).

The identification of an increased frequency of IL-4 releasing T cells in response to IgH-encoded peptides also suggests that such a vaccine can promote a concurrent Th2 immune response. Such a response would be expected to provide helper functions for B-cell activation (Ellyard, Simson et al. 2007). Accordingly, post-vaccine sera from R, but not from NR, contained Abs that specifically recognized autologous neoplastic cells. Therefore, DC-based vaccination may have concurrently induced the production of therapeutic Abs that contributed to the observed clinical responses. The key role of humoral response in controlling the growth of indolent NHLs is also supported by previous results obtained in FL patients after Id-vaccination, which indicated a strong association between the clinical outcome and the induction of a tumour-specific Ab response (Weng, Czerwinski et al. 2004). These data led to the following study on the characterization of the antigens targeted by the humoral responses induced by DC-vaccination in Rs, whose results are described in Chapter 4.

In conclusion, active immunisation with autologous DCs loaded with autologous heat shocked and irradiated tumour cells can provide clinical and immunological efficacy in previously treated indolent NHL patients with measurable disease. However, most of these patients did not respond to this immune intervention, suggesting that it is not always able to generate the required anti-tumour therapeutic Abs and/or the sufficient numbers of effector T cells with the appropriate specificity, functional status, homing properties and survival characteristics to overcome existing tumour escape mechanisms and to promote effective anti-tumour responses in the presence of a significant tumour load (Rosenberg 2004; Park 2008). Therefore, the identification of predictors of the clinical outcome of a cancer vaccine and the development of new strategies to potentiate the immunological and clinical efficacy of DC-based vaccination are required for a better selection of the patients and for improving the clinical benefit associated to this therapeutic intervention. The results described above highlight differences in the immune responses between the Rs and NRs to the autologous anticancer vaccine, which may help identifying those patients who may benefit most from this intervention, indicating areas of further study in the mechanisms of the anti-tumour response. These observations formed the basis of the subsequent work that is described in the following Chapters. In addition, the present study also provided valuable information on the potential to improve vaccine regimens.

3 IMPROVED CLINICAL OUTCOME IN INDOLENT B-CELL LYMPHOMA PATIENTS VACCINATED WITH AUTOLOGOUS TUMOUR CELLS UNDERGOING IMMUNOGENIC DEATH

3.1 Introduction

Vaccination with cancer cells treated *ex-vivo* with anthracyclines, or oxaliplatin, or ionizing radiation protects mice against a subsequent challenge with live tumour cells (Obeid, Tesniere et al. 2007a; Obeid, Tesniere et al. 2007b; Tesniere, Schlemmer 2010). The immunizing properties of killed tumour cells depends on the ability of a cytotoxic agent to render their death immunogenic so that the immune system can be specifically alerted to the presence of a tumour (Zitvogel 2008). Pre-clinical studies of radio/chemotherapy-elicited immunogenic cell death in sarcoma, breast, and colon carcinoma mouse models (Obeid, Tesniere et al. 2007a; Obeid, Tesniere et al. 2007b; Tesniere, Schlemmer 2010; Apetoh 2007) have shown that the release of "eat-me" and danger signals by tumour cells is the main molecular mechanism thereby DC engulfment of dead cell particles and their activation promote the cross-priming of tumour antigens and induction of a specific adaptive immune response (Shi, Evans et al. 2003; Tesniere, Apetoh et al. 2008). Contributing factors to the augmentation of the immune response are described below.
During the early phase of apoptosis, the surface translocation of the endoplasmic reticulum-resident chaperone CRT on dying cells is crucial for their recognition and engulfment by DCs and constitutes the first checkpoint of the immunogenic death process (Obeid, Panaretakis et al. 2007) (Obeid, Tesniere et al. 2007a; Obeid, Tesniere et al. 2007b). Anthracyclines, oxaliplatin and ionizing irradiation induce the rapid translocation of CRT complexed to its molecular partner ERp57 onto tumour cell-surface (Panaretakis, Joza et al. 2008), an event that strongly correlates with effective anti-tumour immunisation in mouse models (Obeid, Tesniere 2007a; Obeid, Tesniere et al. 2007b). The failure to expose CRT after the proper stimulation, thus impairing the immunogenicity of dying tumour cells, has been recognized as an additional mechanism of tumour immune escape (Panaretakis, Joza 2008).

In the late phase of apoptosis and during necrosis, HSP70 and HSP90 can be exposed on the cell surface, where they promote the activation of an immune response by acting as vehicles for peptide antigens (Binder, Srivastava et al. 2005) and providing "eat-me" and danger signals for DCs (Spisek, Charalambous et al. 2007; Somersan, Larsson et al. 2001). In addition, the release from late-apoptotic cells of the non-histone chromatin binding nuclear factor HMGB1 was shown to be indispensable for optimal tumour antigen presentation by DCs (Apetoh, Ghiringhelli et al. 2007). Importantly, TLR4 A896G polymorphism, which weakens HMGB1-TLR4 interactions, affects antigen cross-presentation by DCs and is associated with an unfavourable outcome after antracycline- or oxaliplatin-based regimens in breast and colorectal cancer patients respectively (Apetoh, Ghiringhelli et al. 2007) (Tesniere, Schlemmer et al. 2010). Lastly, the release by dying tumour cells of HMGB1 together with nucleotides, such as ATP and UTP, have shown to participate to the activation in DCs of the "inflammasome" and caspase-1 that lead to the proteolytic maturation and release of IL-1β (Ghiringhelli, Apetoh et al. 2009). These events have been demonstrated decisive for the stimulation of an immune response against dying tumour cells, providing a novel link between the innate and acquired immune systems (Ghiringhelli, Apetoh et al. 2009; Aymeric, Apetoh L, Ghiringhelli F, et al. 2010).

3.2 Aims of the Chapter

Increasing evidence argues that the success of an anticancer treatment may rely upon immunoadjuvant side effects, including the induction of immunogenic tumour cell death. Similarly, the way in which neoplastic cells are killed to produce the antigenic content of a DC-based vaccine may be crucial for priming a clinically efficacious anti-tumour immune response. In the present Chapter, the assumption that the occurrence of immunogenic death was a prerequisite even for the efficacy of an active immunotherapy using autologous DCs loaded with autologous killed tumour cells has been examined. Since vaccination with DCs pulsed with autologous tumour cells dying after exposure to heat shock (HS), γ-irradiation (γ) and UVC-rays (UVC) elicited a clinical response associated with tumour-specific immune-activation in 6 out of 18 relapsed indolent NHL patients, the aim of this study was to determine whether R and NR could be distinguished in terms of immunogenic tumour cell killing at the time of vaccine preparation. Towards this aim, the ability of HS, γ, and UVC to induce CRT and HSP translocation as well as HMGB1 and ATP release was firstly investigated in 3 cell lines, representing low-, intermediate-, and high-grade NHLs. The possibility that a defective exposure of immunogenic signals (Panaretakis, Kepp et al. 2009) by heat shocked, γ-, and UVC-irradiated tumour cells used as antigenic cargo for DC-based vaccination might be associated with a reduced probability of response was investigated. The information obtained finally aimed at identifying predictors of the clinical outcome of a cancer vaccine.

3.3 Materials and Methods

3.3.1 Cell lines, primary cells, and culture conditions

DOHH-2, SU-DHL-6, and SU-DHL-4 cell lines, representing follicular centroblastic/centrocytic, diffuse mixed small and large cell, and diffuse large cell cleaved cell NHL respectively, were purchased from DSMZ (Braunschweig, Germany) and cultured in RPMI 1640 (Lonza, Basel, Switzerland) supplemented with 10% (v/v) inactivated fetal bovine serum (Lonza), 1% (v/v) L-glutamine (Lonza) and 1% (v/v) Hepes buffer (Lonza) in a humidified chamber (95% air, 5% CO2) at 37°C. All cell lines were regularly screened to ensure absence of mycoplasma contamination by MycoAlert® Mycoplasma Detection Kit (Lonza), according to the manufacturer's instructions. Primary lymphoma cells were isolated from LNs or PB as reported in Chapter 2. Written informed consent for the investigational use of lymphocytes, serum samples and tumour specimens was obtained from each patient. Apoptotic and necrotic cell bodies were generated by exposing primary lymphoma cells and cell lines to HS, γ, and UVC in accordance with cell death induction protocol described in Chapter 2, called ALL when compared with the same agents used singly. Lymphoma cells were also treated with DXR (Pfizer Italia s.r.l.) at the indicated doses as the positive control for immunogenic death induction.

3.3.2 Flow cytometry analysis

Surface immunolabelling studies were performed after blocking nonspecific Ab binding to the Fc-receptors within B-cell samples (FcR blocking reagent, Miltenyi Biotec). The following mouse anti-human Abs were used: monoclonal FITC-labelled anti-HLA-DQ, PerCP-labelled anti-HLA-DR (BD Biosciences), PE-labelled anti-HSP70, anti-HSP90, purified anti-CRT (Stressgen, Ann Arbor, MI), and polyclonal anti-HLA class I (clone W6/32, Sera-Lab, Sussex, UK). FITC-labelled goat anti-mouse Igs (Jackson Immunoresearch, Suffolk, UK) was the secondary Ab. To avoid intracellular protein detection, dead cells were excluded by 7-AAD (Invitrogen, Eugene, Oregon, USA) co-staining (1μg/ml for 20 min at 4°C). The immunophenotypic analyses of Tregs and NK cells were determined as described in Chapter 2 in pre- and post-vaccination PB samples from the patients whose immunological monitoring was not part of the previous study. As negative controls, cells were incubated in parallel with the relevant isotype Igs (PE-labelled and purified mouse IgG1, Stressgen; FITC-labelled Rat IgG2a, eBiosciences). The Apoptosis Detection kit (Bender MedSystem, Vienna, Austria) was used in accordance with the manufacturer's protocol.

3.3.3 ELISA

Cellular release of HMGB1 was measured in 24-hour culture supernatants using the appropriate ELISA kit according to the manufacturer's instructions (Shino Test Corporation, Kanagawa, Japan).

3.3.4 ATP release assay

Extracellular ATP was measured in 24-hour culture supernatants by means of the luciferin-based ENLITEN ATP Assay (Promega, Madison, WI). Light emission was recorded with a Berthold luminometer (Berthold Detection systems Gmbh, Pfortzheim, Germany).

3.3.5 Purification and biotinylation of human Igs

Ab purification from human serum and immunoglobulin biotinylation were performed as described in Chapter 2.

3.3.6 Western Blot

DOHH-2 cells or apoptotic and necrotic cell bodies were lysed for 1 hour on ice in lysis buffer (50 mM Tris-HCl pH 7.2, 150 mM NaCl, 100mM NaF, 100mM sodium pyruvate, 1% Triton X-100) containing protease inhibitors (Complete Mini, Roche Applied Science, IN, USA), 2 mM phenylmethylsulfonylfluoride, and 2mM Na3VO4. Protein concentration was determined by the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA). Protein extracts were separated by electrophoresis on precast polyacrylamide gels (Invitrogen, Carlsbad, CA), transferred to hydrophobic polyvinylidene difluoride (PVDF) membranes (Amersham, Pittsburgh, PA), probed with biotinylated human Igs, anti-CRT, -HSP90 and -HSP70 (Stressgen) mAbs or rabbit anti-human actin polyclonal Ab (Sigma, Milan, Italy), and visualized as previously described (Pupa, Argraves et al. 2004). Full-range Rainbow molecular marker (12-225 kDa, Amersham, GE Healthcare) and Sharp Protein Standard (3.5-260 kDa, Novex, Invitrogen) were run in parallel in each sodium dodecyl sulphate - polyAcrylamide gel electrophoresis (SDS-PAGE) analyses. Images were acquired on ArtixScan F1 scanner (Microtek International Inc., Hsin-Chu, Taiwan) using SilverFast Launcher software (MicrotekSDK) and processed with Photoshop CS4 software (Adobe Systems Incorporated, San Jose, CA).

3.3.7 In-gel tryptic digestion, MALDI-TOF-MS analysis, and peptide mass fingerprinting

Protein profiling of DOHH-2 apoptotic and necrotic cell bodies was performed by *the Proteomics Unit* at our Institution. Briefly, protein bands were excised from Coomassie-stained preparative gels and processed as described (Gorla, Mondellini et al. 2009). Matrix-assisted laser desorption/ionization, time-of-flight mass spectrometry (MALDI-TOF-MS) was carried out with a Voyager-DE STR (Applied Biosystems; Milan, Italy), equipped with a nitrogen laser (337 nm). Monoisotopic peptide masses were analysed using Aldente software (http://www.expasy.ch/tools/aldente/). Input was searched according to the following database: Aldente, UniProtKB/SwissProt; predefined taxon: Mammalia; Spectrometer internal error max: 25. Only proteins identified from 3 or more separate experiments were considered.

3.3.8 Gene Ontology analysis

The Database for Annotation, Visualization, and Integrated Discovery (DAVID; http://www.david.niaid.nih.gov) was used to discover the gene ontology biological processes, cellular components, molecular functions (GO terms), and Kyoto encyclopedia of genes and genomes (KEGG) pathways significantly represented by proteins from DOHH-2 apoptotic and necrotic bodies.

3.3.9 Statistical analysis

Statistical significance was calculated using the 2-sided Student's t test ($p \leq 0.05$). Pearson and Spearman correlation coefficients were calculated to measure dependence between variables. Statistical analyses were performed on the Prism 5.0a software version for Macintosh (GraphPad Software, Inc.).

3.4 Results

3.4.1 Immunogenic signals in heat shocked, γ-, and UVC- irradiated NHL cell lines

The extent of CRT cell surface translocation, HMGB1, and ATP release were evaluated in DOHH-2, SU-DHL-6, and SU-DHL-4 cell lines treated with HS, γ, UVC, or ALL. Cells were treated with DXR in parallel as a positive control. The extent of CRT cell surface translocation was directly proportional to the amount of DXR in a limited dose interval (0.1-20 μM), and peaked at 0.5, 2.5, and 15 μM for DOHH-2, SU-DHL-6, and SU-DHL-4, respectively (data not shown). DXR was therefore administered at 0.5, 2.5, and 15 μM in cultures of DOHH-2, SU-DHL-6, and SU-DHL-4 cells respectively, and approximately 50% cell growth inhibition was reached after 24 hours (Figure 3.1A). ALL was highly cytotoxic against the 3 NHL cell lines tested, as shown by the Trypan blue exclusion test and flow cytometry analysis of apoptosis (Figure 3.1A and B).

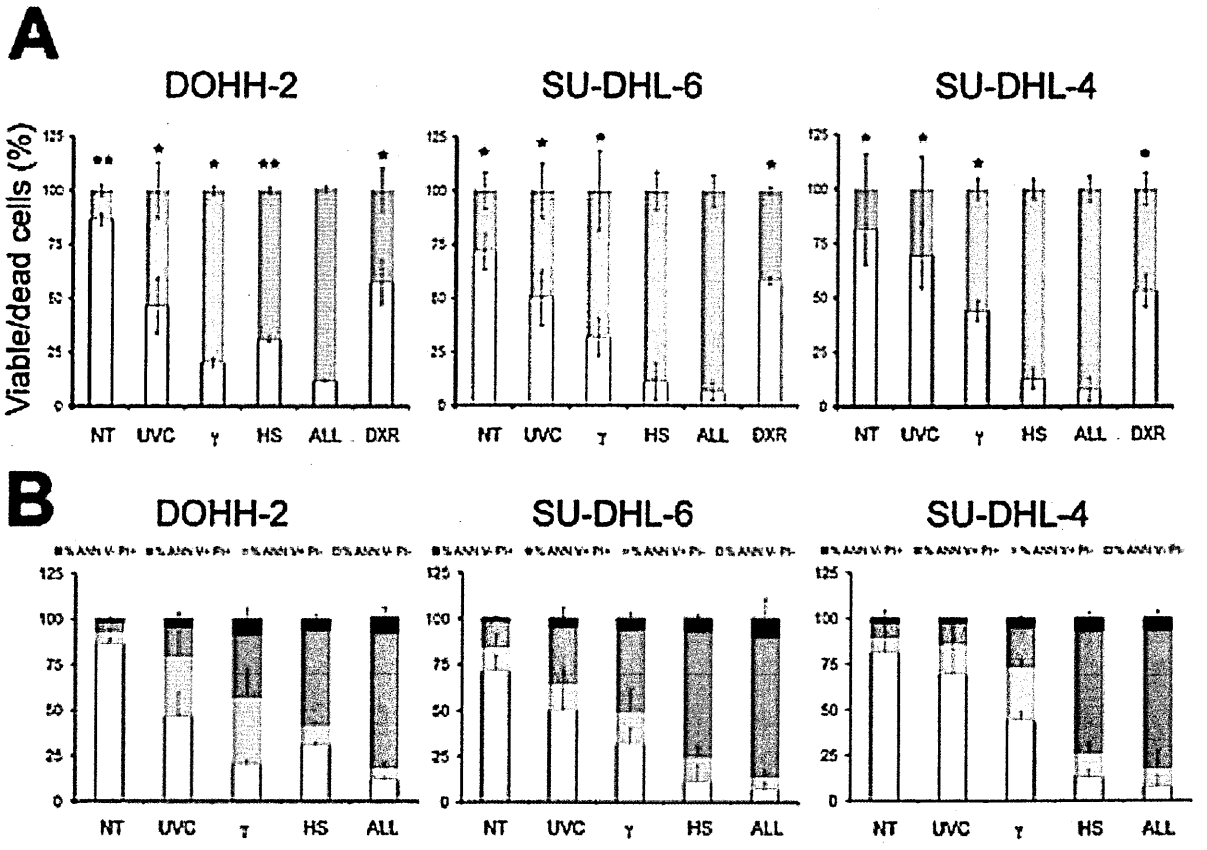


Figure 3.1 Cytotoxic effects of DXR or UVC-rays, γ-irradiation, heat shock or ALL in indolent NHL cell lines.

(A) Percentage of viable (white) and dead cells (grey) as determined by the Trypan blue exclusion test; (B) flow cytometry analyses of apoptosis (Annexin V; PI: propidium Iodide; ANN V-PI-: white columns; ANN V+PI-: light grey columns; ANN V+PI+: dark grey columns; ANN V-PI+: black columns) in DOHH-2, SU-DHL-6, and SU-DHL-4 cells after 24-hour incubation following exposure to UVC- (UVC), γ-irradiation (γ) and heat shock (HS) as single or combined agents (ALL) or let untreated (NT). Significant differences with respect to ALL were calculated using Student *t* test (*$p \leq .05$; **$p \leq .001$; ***$p \leq .0001$). The average values of results obtained in 3 independent experiments are reported.

ALL-treated cells expressed surface-CRT at a higher frequency (Figure 3.2Ai, white columns) and intensity (Figure 3.2Ai, grey columns) compared to cells exposed to γ, UVC or DXR alone. ALL provided a similar amount of CRT cell-surface translocation, regardless of lymphoma histological grade (Figure 3.2Ai, left vs. middle vs. right panel). As additional signals promoting the recognition and uptake of dying cells by DCs, the surface-expression of stress-inducible HSP90 and HSP70 was evaluated. ALL significantly enhanced that of HSP90 in DOHH-2, SU-DHL-6, or SU-DHL-4 compared to γ or UVC alone (Figure 3.2Aii), but that of HSP70 only in SU-DHL-6 cells and to a lesser extent (Figure 3.2Aiii). The ability of DXR and ALL to stimulate cellular release of HMGB1 and ATP, two distinctive signals of immunogenic cell death, was also compared. For each cell line tested, ALL provided a significantly higher release of HMGB1 and ATP from each cell line than DXR administration as revealed by specific ELISA and luciferin-based assays in 24-hour conditioned culture media (Figure 3.2Bi, p=0.045, p=0.029, p=0.0002; Figure 3.2Bii, p=0.049, p=0.045, p=0.024). Interestingly, CRT and HSP90 exposure in heat-shocked NHL cell lines was comparable to that achieved in ALL-treated cells (Figure 3.2Ai-ii); however, HS alone was less efficient than ALL to boost ATP and HMGB1 release from all 3 cell lines (data not shown).

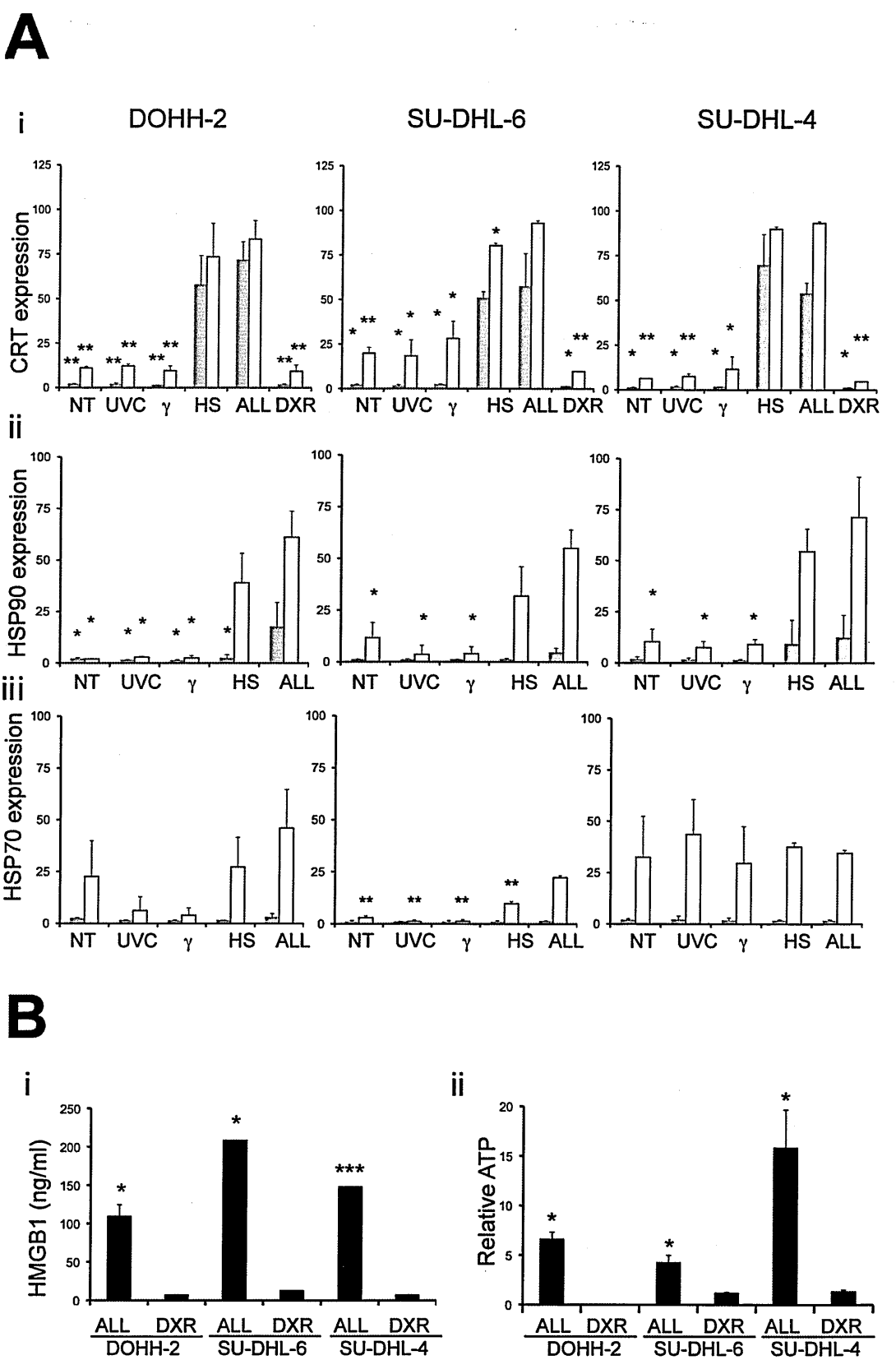


Figure 3.2 Immunogenic signals in treated NHL cell lines.
(A) Flow cytometry analyses of CRT (i), HSP90 (ii) and HSP70 (iii) relative median fluorescence intensity (MFI, grey columns) and positive cell frequency (white columns). Relative MFI was calculated as the ratio between stained sample and negative control MFI. Analyses were performed on 7-AAD negative-gated events. (B) Quantification of HMGB1 (i) and ATP (ii) in culture supernatant. For each cell line, ATP release was normalized by the amount found in untreated cultures, giving relative ATP values. Statistically significant differences between ALL and the other culture conditions were calculated using the 2-sided Student *t* test The average values of results obtained in 3 independent experiments are reported.

3.4.2 Analyses of protein changes occurring as a result of "ALL" treatment

The combination of γ and UVC with HS (ALL) was thus an effective way of inducing all reported mediators of immunogenic cell death in 3 differently aggressive NHL cell lines. Coomassie staining revealed that ALL modified patterns of proteins present in lysates from the DOHH-2 cell (Figure 3.3A, B). These proteins were subjected to mass spectrometry (MS) and the results are shown in Table 3.1.

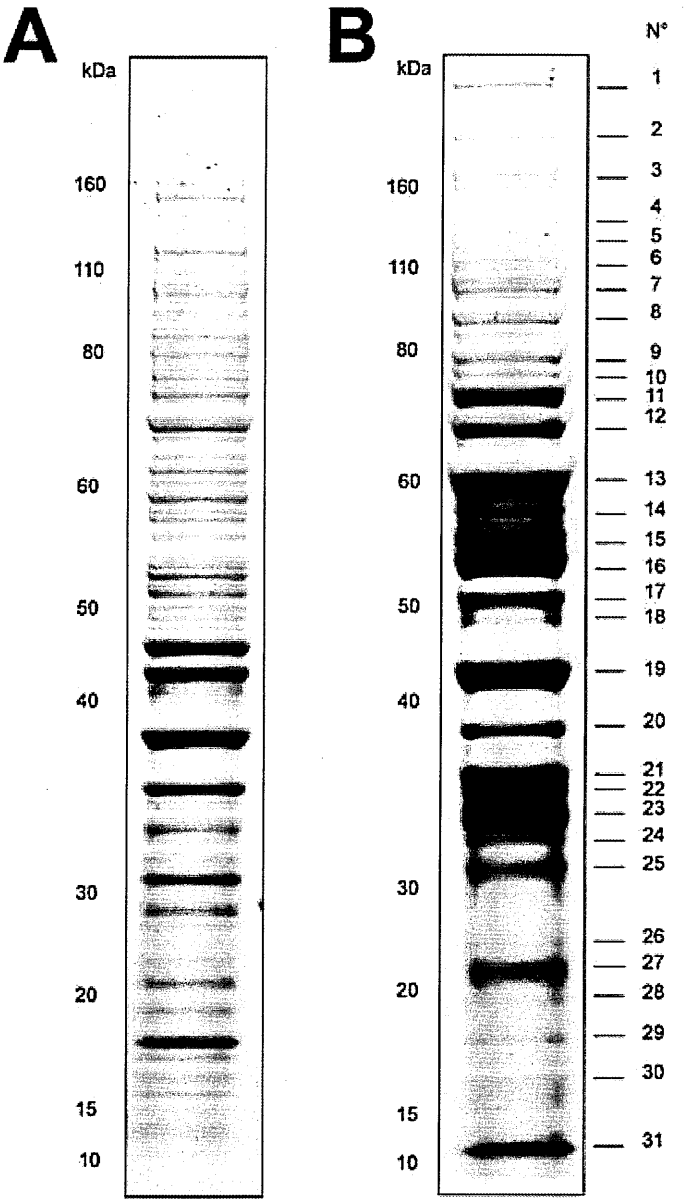


Figure 3.3 Coomassie staining of DOHH-2 cells and apoptotic bodies.
(A) SDS-PAGE of live and (B) ALL-treated DOHH-2 cell protein extracts revealed by Coomassie staining. N°: identification number of bands analysed by MS as reported in Table 3.1.

Table 3.1 List of MS-identified proteins in bands indicated in Figure 3.3B.
N°. *,† Swiss-Prot/TrEMBL accession and description line; ‡,§theoretical protein mass and isoelectric point; ||peptide/protein amino acid numbers.

N°	AC*	DE†	Mw‡	pI§	Cov||
1	P35579	Myosin-9	226	5.5	32
2	Q00610	Clathrin heavy chain 1	191	5.5	32
	Q9NYU2	UDP-glucose ceramide glucosyltransferase-like 1	173	5.4	17
	P53675	Clathrin heavy chain 2	187	5.6	12
3	Q9Y4L1	Hypoxia up-regulated protein 1	108	5.1	26
	Q96F07	Cytoplasmic FMR1-interacting protein 2	148	7	18
	P35579	Myosin-9	226	5.5	13
4	P53396	ATP-citrate synthase	121	7	18
5	Q14697	Neutral alpha-glucosidase AB	104	5.6	28
	Q02218	Alpha-ketoglutarate dehydrogenase	111	6.1	26
	Q9NSE4	Isoleucyl-tRNA synthetase, mithocondrial	109	6.2	23
6	Q14697	Neutral alpha-glucosidase AB	104	5.6	39
	O43707	Alpha-actinin-4	105	5.3	26
7	P13639	Elongation factor 2	95	6.4	31
	P14625	Endoplasmin	90	4.7	27
	P05023	Sodium/potassium-tramsporting ATPase sub	112	5.3	20
8	P08238	**Heat shock protein HSP90-beta**	83	5	41
	P07900	**Heat shock protein HSP90-alpha**	85	5	39
9	P27824	Calnexin	65	4.5	26
10	Q12931	Heat shock protein 75 kDa, mitochondrial	74	6.1	42
	P11021	78 kDa glucose-regulated protein	70	5	31
	P52272	Heterogeneous nuclear ribonucleoprotein M	77	8.8	27
11	Q12931	Heat shock protein 75 kDa, mithocondrial	74	6.1	50
	P11021	78 kDa glucose-regulated protein	70	5	39
	P38646	Stress-70 protein mithocondrial	69	5.5	31
	P26038	Moesin	68	6.1	27
12	P04843	Dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit 1	66	6	49
	P13796	Plastin-2	70	5.2	42
	P31040	Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial	68	6.2	40
13	P10809	60 kDa heat shock protein, mitochondrial	58	5.2	43
	P14618	Pyruvate kinase isozymes M1/M2	58	8	33
14	P00367	Glutamate dehydrogenase 1, mitochondrial	56	6.7	38
	P30101	**ERp57**	54	5.6	36
15	P25705	ATP synthase subunit alpha, mitochondrial	55	8.3	57
	P00367	Glutamate dehydrogenase 1, mitochondrial	56	6.7	31
16	P06576	ATP synthase subunit beta, mitochondrial	52	5	56
	P34897	Serine hydroxymethyltransferase, mitochondrial	53	8.1	47
17	P31930	Cytochrome b-c1 complex subunit 1, mitochondrial	49	5.4	50
	P06733	Alpha-enolase	47	7	43
18	P61158	Actin-related protein 3	47	5.6	38
19	P60709	Actin, cytoplasmic 1	42	5.3	46
	P63261	Actin, cytoplasmic 2	42	5.3	42
	P00505	Aspartate aminotransferase, mitochondrial	45	9	33
20	P04406	Glyceraldehyde-3-phosphate dehydrogenase	36	8.6	38
21	Q99623	Prohibitn-2	33	9.8	68
	P40926	Malate dehydrogenase, mithocondrial	33	8.5	69
	P63244	Guanine nucleotide-binding protein subunit beta-2-like 1	35	7.6	67
22	Q99623	Prohibitn-2	33	9.8	63
23	P05141	ADP/ATP translocase 2	33	9.8	56
	P12236	ADP/ATP translocase 3	33	9.8	49
	P12236	ADP/ATP translocase 1	33	9.8	47
	Q00325	Phosphate carrier protein, mitochondrial	35	9.3	37
	Q13011	Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase, mitochondrial	32	6	52
24	P35232	Prohibitin	30	5.5	57
25	P18669	Phosphoglycerate mutase 1	29	6.8	46
26	P24539	ATP synthase subunit b, mithocondrial	25	9.1	36
27	P23284	Peptidyl-prolyl cis-trans isomerase B	20	9.2	50
	P48047	ATP synthase subunit O, mithocondrial	21	9.8	58
28	P62937	Peptidyl-prolyl cis-trans isomerase A	18	7.8	62
	P62269	40S ribosomal protein S18	18	11	36
29	Q04837	Single-stranded DNA-binding protein, mitochondrial	15	8.2	77
30	Q04837	Single-stranded DNA-binding protein, mitochondrial	15	8.2	69
	P07737	Profilin-1	15	8.5	48
31	P56134	ATP synthase subunit f, mithocondrial	11	9.7	31

MS-based multiplexed profiling of apoptotic/necrotic DOHH-2 cell bodies highlighted an enrichment in proteins involved in mitochondrial and endoplasmic reticulum functions, ATP metabolism, intracellular organelle reorganization, and stress response (Table 3.1), which are represented in 15 gene ontology (GO) categories (Table 3.2). Seven KEGG pathways, including oxidative phosphorylation, citrate cycle, and antigen processing and presentation were also significantly represented in ALL-treated DOHH-2 cells (Table 3.3). Most of the functions and pathways detected were integrally involved in apoptosis and in biochemical events associated with the immunogenicity of dying cells, and thus emphasized the ability of HS associated with γ and UVC to generate immunogenic apoptotic and necrotic bodies from NHL cell lines.

Table 3.2 Gene Ontology (GO) of proteins significantly represented in DOHH-2 apoptotic and necrotic bodies.

GO Terms	Fold Enrichment	*p*
Endoplasmic reticulum	1.87	1.36E-002
Actin cytoskeleton organization	1.95	1.12E-002
Heat-shock proteins (HSP70)	2.8	1.47E-003
Intracellular transport	3	1.05E-003
ATP carrier protein	3	1.02E-003
ATP biosynthesis	3.4	3.95E-004
UPR/stress response	3.7	1.70E-004
Localization/transport	4	9.31E-005
Heat-shock proteins (HSP90)	4.1	7.38E-005
Catabolic processes	4.1	7.27E-005
ATP binding	4.6	2.20E-005
Monosaccharide metabolism	5	8.89E-006
Intracellular	5.9	1.24E-006
Vesicle	6.8	1.60E-008
Mitochondrion	12.6	2.61E-013

Table 3.3 KEGG pathways significantly represented by ALL-treated DOHH-2 cells.

Kegg Pathway	%	Fold Enrichment	*p*
hsa04810: Regulation of actin cytoskeleton	10.53	2.9	4.99E-002
hsa00190: Oxidative phosphorylation	12.28	5.6	1.15E-003
hsa04612: Antigen processing and presentation	8.77	6.4	6.55E-003
hsa00010: Glycolysis/Gluconeogenesis	7.02	6.9	1.87E-002
hsa05040: Huntington's disease	5.26	10.6	3.04E-002
hsa00710: Carbon fixation	5.26	13.4	1.96E-002
hsa00020: Citrate cycle (TCA cycle)	7.02	13.7	2.69E-003

3.4.3 Immunogenic cell-death in primary indolent NHL exposed to heat shock, γ- and UVC- rays

The results obtained in long-term NHL cell lines led to investigate whether combined exposure to HS, γ, and UVC was the best way to induce immunogenic cell death in primary B-NHLs. Because ALL constituted the cell death protocol adopted to kill indolent NHL primary cells and obtain tumour antigen cargo for patient-specific anti-lymphoma DC-based vaccines, as shown in Chapter 2, the surface exposure of CRT and HSPs and the release of HMGB1 were analysed in treated FL cells isolated from 4 patients (Table 3.4, patients #1, 10, 11, 14) before immunotherapy. After 24 hours, ALL killed over 90% of tumour cells as revealed by flow cytometry analysis of apoptosis and reached statistical significance when compared to UVC or HS alone (Figure 3.4A; p=0.049, p=0.047). However, for each treatment condition no significant differences were found between tumour cells from Rs and NRs in terms of amount of cell death (Figure 3.4B).

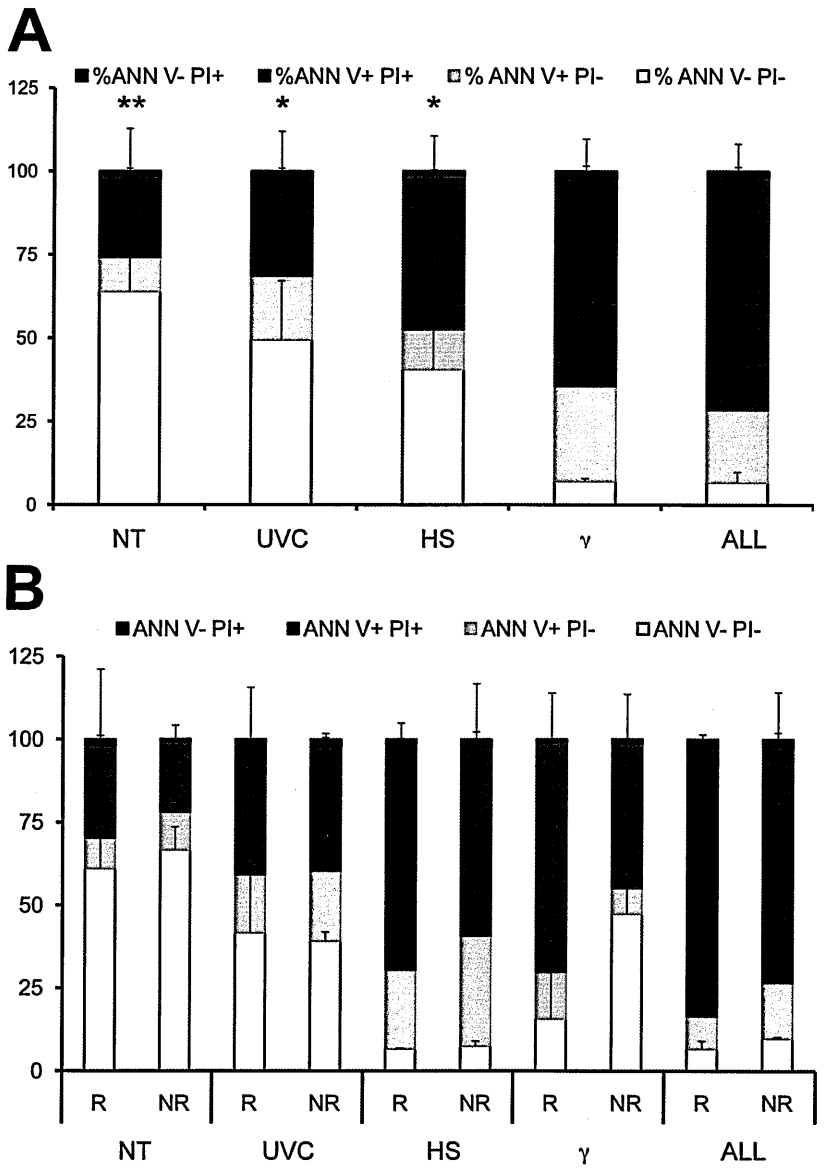


Figure 3.4 Cytotoxic effects of UVC-rays, γ-irradiation, heat shock or ALL in primary indolent NHL cells. Flow cytometry analysis of apoptosis (ANN V: Annexin V; PI: propidium iodide) in differently treated primary FL cells (NT: untreated; UVC-rays: UVC; HS: Heat Shock; γ: γ-irradiation; ALL: HS, γ and UVC) from 4 vaccinated patients (panel A: average patients #1, 10, 11, 14; panel B: average R #1 and 14 vs. NR #10 and 11). Significant differences with respect to ALL were calculated using Student t test (*$p \leq .05$; **$p \leq .001$).

Because only a trend toward the increase of CRT and HSP90 surface expression was observed in ALL as compared with single agent-treated tumours (average of 4 FL samples, data not shown), the ability of dying FL cells to determine these parameters was assessed in samples from Rs (patients #1, 14) and from NRs (patients #10, 11), separated into 2 groups. The extent of CRT exposure on ALL treated tumour cells from Rs was significantly higher compared to the same samples exposed to single agents, as revealed by flow cytometry analysis of the relative MFI (Figure 3.5A, white columns; p=0.022, p=0.037, p=0.024). ALL significantly increased CRT and HSP90 surface expression in tumour cells from Rs as compared with NRs (Figure 3.5A, B white vs. grey columns, p=0.006, p=0.045). No statistically significant differences were found when HSP70 exposure or HMGB1 release were analysed (data not shown).

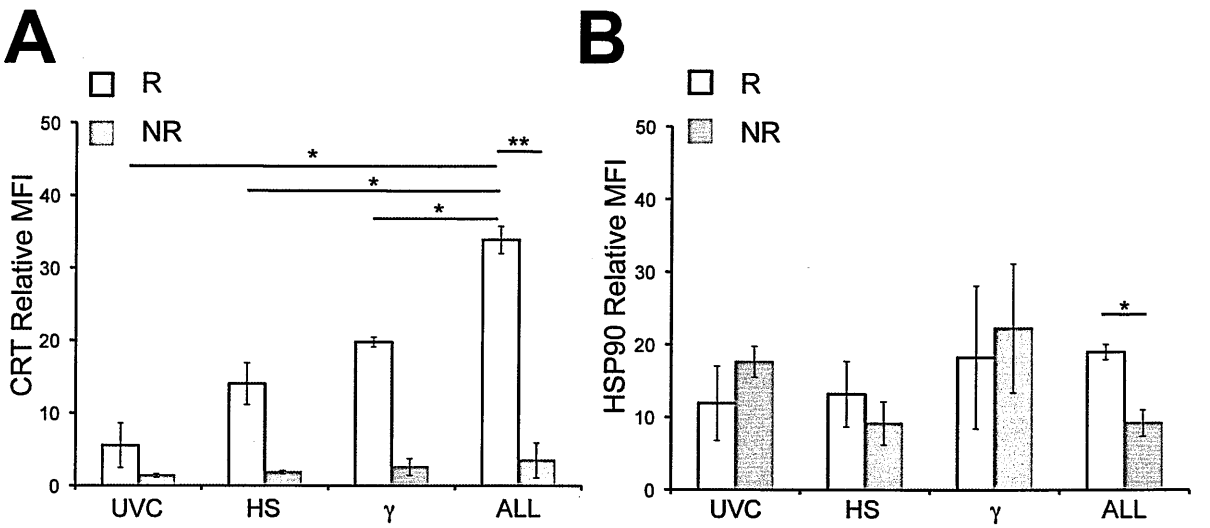


Figure 3.5 CRT and HSP90 expression on treated tumour cells from vaccinated patients.
Flow cytometry analyses of CRT (A) and HSP90 (B) relative MFI in tumour cells from 2 R (average patients #1 and 14, white columns) and 2 NR (average patients #10 and 11, grey columns), 24 hours after exposure to UVC-rays (UVC), heat shock (HS) and γ-irradiation (γ) as single or associated treatments (ALL). Relative MFI was calculated as the ratio between stained sample and negative control MFI of high FSC and low SSC-gated events. Significance was calculated using the 2-sided Student t test (*$p \leq .05$; **$p \leq .001$).

These results indicate that equally treated primary FL, with a similar clinical history, may differ in their ability to emit immunogenic signals.

3.4.4 Humoral response to immunogenic cell death markers

To determine whether the immunisation efficacy of a DC-based vaccine depends on the extent of immunogenic signal exposure by dying tumour cells loaded on DCs, the presence of anti-CRT and -HSP90 Abs was verified in pre- and post-vaccination serum samples. Apoptotic and necrotic DOHH-2 cells were used as target cells in western blot analyses, because upon exposure to ALL they displayed surface CRT and HSP90 (Figure 3.2Ai, Aii) as well as a significant proteome enrichment of HSP90 and Erp57, the cognate functional partner of CRT (Panaretakis, Joza et al. 2008) (Table 3.1). Protein extracts were probed with biotin-conjugated Igs purified from pre- and post–vaccination serum samples of Rs (Figure 3.6Ai, ii, Figure 3.6Bi, ii) and NRs (Figure 3.6Aiii, iv, Figure 3.6Biii, iv) or commercial anti-CRT (Figure 3.6Av) and -HSP90 (Figure 3.6Bv) mAbs. Remarkably, Rs showed the presence of a greater amount of circulating Abs directed against proteins migrating at molecular weights compatible with CRT (Figure 3.6Ai, ii vs. 3Av) and HSP90 (3.6Bi, ii vs. 3Bv) compared to NRs (Figure 3.6Aiii, iv vs. 3.6Av and Figure 3.6Biii, iv vs. 3.6Bv). This provided evidence for a positive association between tumour cell ability to

expose immunogenic signals and their immunizing properties when loaded into DCs and injected into these two patients.

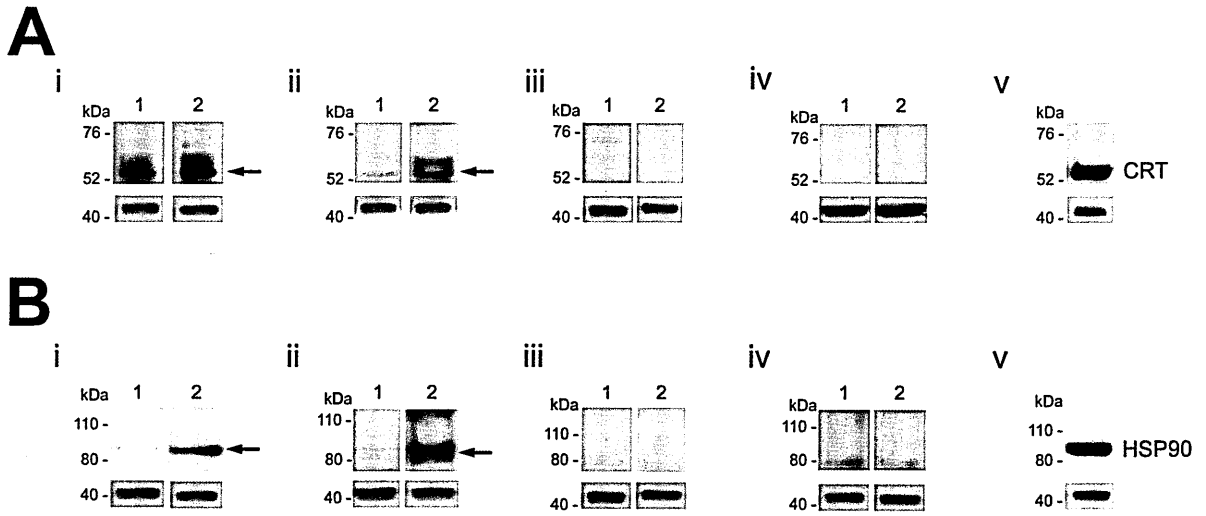


Figure 3.6 Vaccine-specific humoral response in clinical Rs.
Western blot analyses of patients' Igs on DOHH-2 apoptotic and necrotic body extracts. (A) Reactive bands obtained by probing with pre- (lane 1, upper) or post- (lane 2, upper) vaccine Igs from 2 Rs (i, patient #1; ii, patient #14) and 2 NRs (iii, patient #10; iv, patient #11) or with anti-CRT commercial mAb (v, upper). (B) Reactive bands obtained by probing with pre- (lane 1, upper) or post- (lane 2, upper) vaccine Igs from R (i, patient #1; ii, patient #14) and NR (iii, patient #10; iv, patient #11), or with anti-HSP90 commercial mAb (v, upper). Actin protein expression is reported at the bottom of each panel as loading control. Arrows indicate specific bands for CRT and HSP90.

3.4.5 Immunogenic tumour cell death and clinical efficacy of vaccination with killed autologous NHL cell-pulsed DCs

To determine the involvement of immunogenic tumour cell death in the efficacy of DC-based active immunotherapy in indolent NHL patients, the ability of tumour cells to provide immunogenic signals on exposure to ALL was studied in all samples still available from vaccinated patients (Rs=6; NRs=8) according to their outcome after vaccination (Table 3.4). Whereas the extent of cell death did not differ between the two groups (Figure 3.7A, p=0.127), CRT positive cell percentage and MFI were significantly higher in dying tumour cells from Rs compared to NRs, as revealed by flow cytometry (Figure 3.7B, top left p=0.023 and bottom left p=0.039). Furthermore, apoptotic and necrotic bodies from tumour cells of Rs expressed surface HSP90 at a higher frequency and intensity compared to those from NRs (Figure 3.7B, top middle, p=0.001 and bottom middle, p=0.002); no significant differences were found when HSP70 was analysed (Figure 3.7B, top right p=0.421 and bottom right p=0.481). Flow cytometry analyses of HLA class I and II expression on dying tumour cells from Rs and NRs revealed no significant differences (data not shown), suggesting that they shared similar antigen presentation properties. Similarly, HMGB1 release from dying tumour cells did not differ between Rs and NRs (Figure 3.7C, p=0.705).

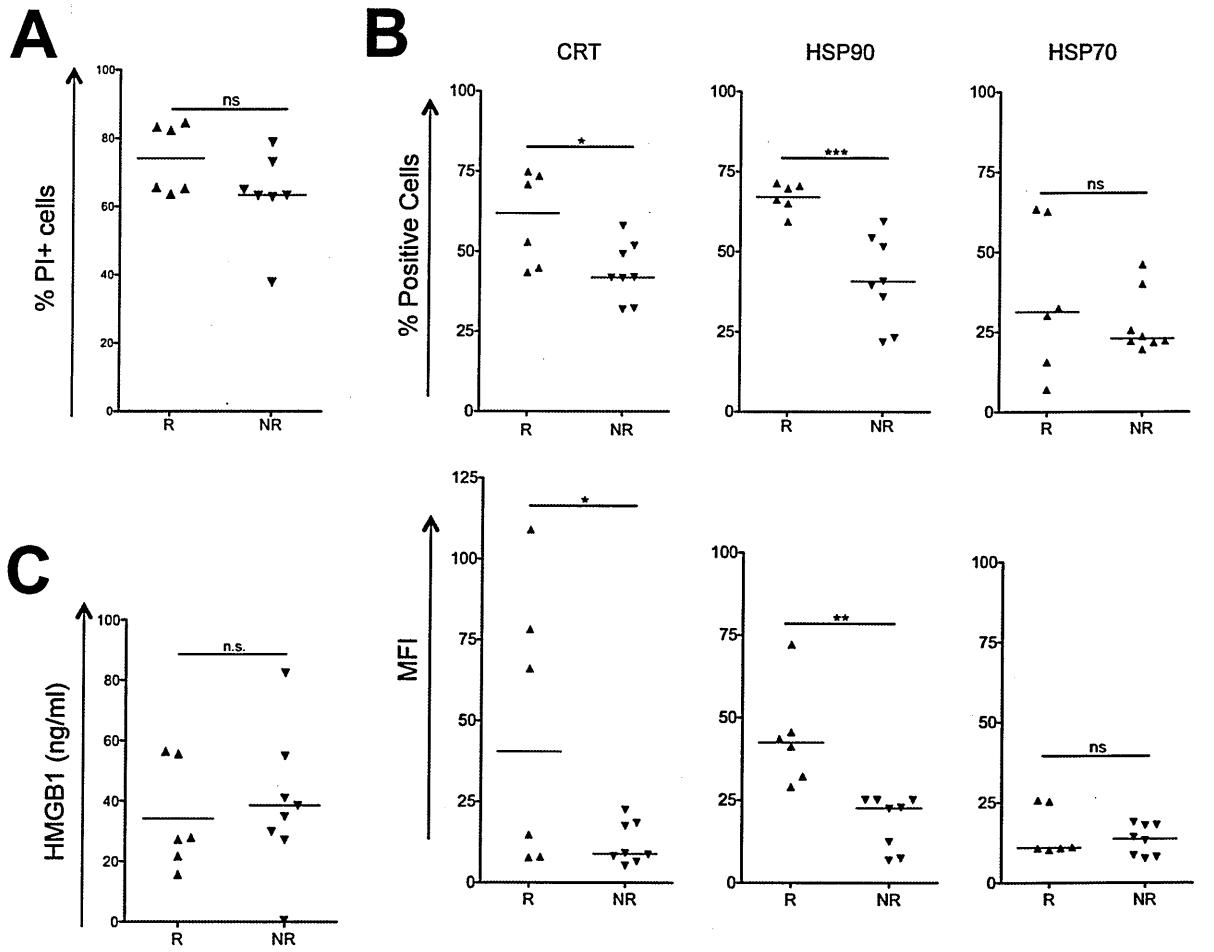


Figure 3.7 Lethal and immunogenic response in tumour cells from Rs and NRs.
Twenty-four hours following exposure to ALL, the ability of tumour cells from Rs and NRs to undergo immunogenic death was assessed. Flow cytometry analyses of (A) propidium iodide+ tumour cell frequency (% PI+ cells); (B) positive cell frequency (upper) and MFI (lower) for CRT (left), HSP90 (middle) and HSP70 expression (right); and (C) quantification of HMGB1 release in culture supernatant from apoptotic and necrotic tumour cells. Significance was calculated using the 2-sided Student *t* test (ns= non significant difference; $*p \leq .001$; $**p \leq .001$; $***p \leq .0001$).

The strength of the correlation between the extent of CRT and HSP90 exposure on killed autologous tumour cells used to load DCs for vaccine preparation and patient outcome after vaccination was then measured. The results are summarised in Table 3.4. HSP90 surface-expression in dying tumour cells was found to be significantly associated with the occurrence of clinical responses (HSP90 MFI, Spearman r=0.8631, $p<0.0001$; %HSP90+ cells, Spearman r=0.8235, $p=0.0003$). The frequency of tumour cells expressing surface-CRT also positively correlated with a favourable outcome after vaccination (Spearman r=0.6087; $p=0.0209$). As reported in Chapter 2, Rs showed tumour-specific T- and B-cell activation, the reduction of Treg frequency and NK cell maturation, indicating a potential association between the exposure of immunogenic signals and the ability of a DC-based vaccine to induce a clinically efficient immune activation. Accordingly, clinical responses were significantly associated with NK cell maturation (Spearman r=0.7835, $p=0.0015$) and Treg frequency reduction (Spearman r=-0.7835, $p=0.0015$), which, in turn, were found to directly correlate with the extent of CRT and HSP90 expression in the vaccine antigenic cargo (CRT MFI vs. NK maturation: Pearson r=0.8321, $p=0.0004$; %CRT+ cells vs. NK maturation: Pearson r=0.7506, $p=0.0031$; CRT MFI vs. Treg frequency reduction: Pearson r=-0.6594, $p=0.0142$; HSP90 MFI vs. Treg frequency reduction: Pearson r=-0.6164, $p=0.0249$; %HSP90+ cells vs. Treg frequency reduction: Pearson r=-0.6716, $p=0.0119$).

Table 3.4 Clinical and immunological characteristics of vaccinated patients.

UPN #	Age /Sex	NHL Type, Stage	Previous Treatment (Response/Duration)	Clinical Response (Length in Months)	Immunological Responses			Immunogenic Signals in Dying Tumour Cells			
					Post/Pre Vax Activated NK Cell Frequency[ll]	Post/Pre Vax Anti-tumour T Cells**	Post/Pre Vax Treg Frequency[§]	% CRT[+]	CRT MFI	% HSP90[+]	HSP90 MFI
12	65/ M	LP, Stage IA	6 CVP (CR/36 months)	CR (67)	1.08	n.a.	0.82	70.87	66.10	69.74	41.30
13	72/F	FL grade I, Stage IIA	6 CVP (PR/16 months); 4 Rituximab (CR/24 months)	CR (64)	1.37	n.a.	0.85	74.84	109.00	71.39	72.15
14	52/F	FL grade IIIa, Stage IVA	6 R-MegaCEOP (PR/10 months); HDS (CR/6 months)	CR (63)	2.64	15*	0.16	52.91	14.80	70.50	29.00
1	49/F	LP, Stage IVA	6 CVP (CR/48 months); 8 Rituximab (PR/72 months)	PR (47)	0.99	3.02	0.70	43.44	7.98	62.16	43.60
5	52/ M	FL grade I, Stage IVA	6 CVP (CR/24 months); 4 Rituximab (PR/15 months)	PR (12)	1.11	4.84	0.74	44.83	7.80	66.31	32.25
6	45/ M	FL grade II, Stage IVA	6 R-CEOP (CR/12 months); 4 Rituximab (PR/8 months); HDS (CR/12 months)	PR (7)	1.14	4.75	1.16	73.53	78.20	59.40	45.60
4	51/ M	FL grade II, Stage IA	4 Rituximab (CR/24 months); RT 30 Gy (CR/24 months)	SD (78)	n.a.	1.21	n.a.	58.03	22.50	21.82	6.81
9	63/ M	LP, Stage IVA	8 Rituximab (PR/16 months); 4 CVP (PR/18 months); 4 R-CHOP (SD/32 months)	SD (69)	0.62	1.01	1.18	49.15	5.26	51.50	22.80

UPN #	Age /Sex	NHL Type, Stage	Previous Treatment (Response/Duration)	Clinical Response (Length in Months)	Immunological Responses			Immunogenic Signals in Dying Tumour Cells			
					Post/Pre Vax Activated NK Cell Frequency[II]	Post/Pre Vax Anti-tumour T Cells**	Post/Pre Vax Treg Frequency[§]	% CRT[+]	CRT MFI	% HSP90[+]	HSP90 MFI
10	54/ M	FL grade I, Stage IVA	8 R-CVP (CR/36 months)	SD (68)	1.06	1.13	1.13	31.88	17.48	35.90	22.45
11	72/ M	FL grade II, Stage IVB	18 mo Leukeran (CR/84 months)	SD (10)	0.57	0.89	1.43	32.21	6.50	59.44	25.10
18	62/F	FL grade I, Stage IIIA	6 R-CVP (CR/36 months); 4 Rituximab (CR/26 months)	SD (52)	1.00	0.98	1.56	51.76	18.40	39.46	25.10
7	50/ M	FL grade I, Stage IVA	6 CHOP (PR/12 months); 4 Rituximab (PR/6 months)	PD	0.86	1.05	1.57	41.92	9.07	40.82	12.40
8	56/ M	FL grade II, Stage IA	3 CHOP-bleo/3 CVP (RC/16 months) HDS (CR/24 months); 8 Rituximab (CR/6 months)	PD	0.76	0.85	0.90	41.70	8.61	23.14	7.38
15	72/ M	LP, Stage IA	3 CHOP (PR/8 months); Splenectomy + RT (PR/12 months); 12 months Leukeran (PR/7 months); HDS (CR/12 months); 4 Rituximab (PD/n.a.)	PD	0.84	0.91	1.33	41.67	8.15	54.30	25.10

UPN: Unique Progressive Number; LP: lymphoplasmocytic lymphoma; CR: complete remission, PR: partial response, SD: stable disease, PD: progressive disease, according to Cheson and co-workers (Cheson, Pfistner et al. 2007); CVP: cyclophosphamide, vincristine and prednisone; R-CVP: Rituximab plus CVP; CHOP: cyclophosphamide, adryamicin, vincristine and prednisone; R-CHOP: Rituximab plus CHOP; RT: Radiotherapy; CEOP: cyclophosphamide, epiadryamicin, vincristine and prednisone; R-CEOP: Rituximab plus CEOP; HDS: High dose sequential chemotherapy; LN: Lymph-nodes; BM: Bone marrow; PB: Peripheral blood; tx: Therapy; n.a.: not assessable.

II : Post/pre vaccination ratio of activated NK cell frequency measured by FACS analysis of CD16 expression in CD3-CD56dim-gated cells in Chapter 2.

**: Post/pre vaccination ratio of anti-tumour T cell frequency at tumour site measured by IFN-γ ELISPOT assay in Chapter 2.

* : Post/pre vaccination ratio of idiotype-specific T cell frequency in PB measured by IFN-γ ELISPOT assay in Chapter 2.

§ : Post/pre vaccination ratio of Treg frequency measured by FACS analysis of CD25+FOXP3+ in CD3+CD4+-gated T cells in Chapter 2.

3.5 Discussion

This study confirms, in a human setting, recent findings obtained in mice concerning the possibility of inducing immunogenic death in cancer cells in order to provide a reliable source of antigens for anti-tumour vaccination (Obeid, Tesniere et al. 2007a; Obeid, Tesniere et al. 2007b). The exposure to HS, γ, and UVC in 3 human NHL cell lines representing low-, intermediate- and high-grade lymphomas provided all of the key features required to trigger a DC-mediated anti-tumour immune response. These include the plasma membrane translocation of CRT and HSPs, and the release of HMGB1 and ATP (Tesniere, Apetoh et al. 2008; Aymeric, Apetoh et al. 2010). Because these events were enhanced when the three agents were co-administered, it is necessary to combine HS, γ, and UVC to efficiently induce an immunogenic tumour cell death. This combination was also more effective than DXR at inducing surface translocation of "eat-me" signals coupled with the release of HMGB1 and ATP.

Apoptotic and necrotic tumour cell bodies generated through the exposure of primary indolent NHL cells to HS, γ, and UVC were used to pulse autologous monocyte-derived DCs and thus produce a patient-tailored vaccine. Interestingly, although displaying the same level of apoptosis, necrosis, and HMGB1 release, primary lymphoma samples from Rs demonstrated an improved ability to translocate CRT and HSP90 to the cell surface upon exposure to the combined treatment when compared to NRs patients. Remarkably, vaccination with DCs pulsed with ALL-treated autologous tumours induced the production of circulating anti-CRT and -HSP90 Abs in Rs, but not in NRs, and elicited clinical responses strongly associated with multifaceted anti-tumour immune activation, as described in Chapter 2. These observations, in an admittedly small number of cases, point to a positive correlation between the surface-expression of CRT or HSP90 in dying tumour cells used as antigen cargo for a DC-based vaccine and immunological responses associated with clinical benefit.

These results are in line with those obtained by Zitvogel and colleagues showing that the failure of one early step towards immunogenic cancer cell death was sufficient to abrogate the process (Tesniere, Apetoh et al. 2008). The lack of the CRT exposure alone, for example, may explain the hampered ability of cisplatin to induce immunogenic death in mouse colon cancer cells as compared with oxaliplatin, and thus reflect its limited therapeutic efficacy in colon carcinoma patients (Tesniere, Schlemmer et al. 2010). In this study, instead, the same treatment resulted in a different exposure of "eat-me" signals in clinically comparable primary indolent NHLs, whilst reproducibly providing human NHL cell lines of different histological grades with these immunogenic molecules. Similarly, HS exposure alone was sufficient to kill most of the cells in the 3 NHL cell line cultures and boost their translocation of CRT and HSP90, whereas it was less capable of eliciting the same effects in primary tumours. Alterations in the molecular pathways for chaperone trafficking (Panaretakis, Kepp et al. 2009), which render tumour cell death immunogenically silent, may confer survival advantages to B-NHLs *in vivo* and may be selected as new immune-escape mechanisms. Therefore primary tumour cells may be less prone to undergo immunogenic cell death compared to *in-vitro* established cell lines. Accordingly, the ability to down-regulate CRT expression has been shown to be associated with a negative prognostic/predictive impact in colon cancer (Toquet, Jarry et al. 2007), neuroblastoma (Hsu, Hsieh et al. 2005), and cervical carcinoma (Mehta, Jordanova et al. 2008), as well as in follicular thyroid carcinoma (Netea-Maier, Hunsucker et al. 2008). Nevertheless, treated NHL cell lines in the current study consistently showed CRT and HSP90 cell surface translocation to be distinctive features induced by the combined exposure to HS, γ, and UVC. CRT involvement in immunogenic tumour cell death is also illustrated by the investigation of its immunostimulatory properties, including its ability to elicit a CTL response against chaperoned antigens (Nair, Wearsch et al. 1999) or its own

antigenic epitopes (Lieu, Newkirk et al. 1988) as well as to induce specific auto-Abs in a variety of autoimmune diseases (Eggleton, Ward et al. 2000). HSP expression on the surface of dying tumour cells similarly improves their recognition by DCs (Saito, Dai et al. 2005), and increases the efficient cross-presentation of tumour-derived chaperoned antigenic peptides (Srivastava 2005) and DC maturation (Somersan, Larsson et al. 2001) (Spisek, Charalambous et al. 2007).

Chaperone rich tumour cell lysates activate NK cell effector functions in the presence of accessory cells such as DCs (Zeng, Chen et al. 2006). Chaperone proteins may thus be endowed with a key role in the cross-talk between DCs and NK cells (Zeng, Graner et al. 2006). By killing tumour cells, activated NK cells may render tumour antigens available for further DC cross-presentation *in vivo* (Osada, Clay et al. 2006; Woo, Clay et al. 2006; Bigley, Spence et al. 2010), whilst inhibiting peripheral Treg conversion and directly lysing Tregs (Brillard, Pallandre et al. 2007; Roy, Bames et al. 2008). Regression of the tumour mass may therefore result in re-establishment of the balance between immunity and tolerance in favour of tumour immune surveillance (Yang, Novak et al. 2006). A favourable clinical outcome after vaccination was found indeed to be significantly associated with the extent of chaperone protein expression on apoptotic and necrotic tumour cell bodies loaded into DCs, and, in turn, with NK cell activation and Treg frequency reduction. In keeping with the extensively described property of immunogenic tumour cell death to elicit specific T cell responses (Obeid, Tesniere et al., 2007a; Obeid, Tesniere et al. 2007b; Tesniere, Apetoh et al. 2008), anti-tumour adaptive cellular immunity was detected at the tumour site in PRs, and in PB in one CR, for whom a tumour-specific idiotype T cell response was assessed.
All Rs showed an increased ability to translocate HSP90 as compared to NRs, whereas 3 Rs displayed comparable ability in CRT exposure with respect to NRs, meaning that at least one "eat-me" signal was sufficient to provide the proper stimuli for DC antigen uptake and activation. The considerable expression of HSP90 in vaccines administered in all Rs may have compensated for the limited release of CRT in 3 of them.

These results delineate new ways of optimizing anticancer vaccines for the stimulation of a therapeutic anti-neoplastic immune response. Pulsing of DCs *ex vivo* with killed tumour cells avoids their physiological clearance by neighbouring cells before entering the late stages of the apoptotic process (Savill, Dransfield et al. 2002), and thus ensures optimal DC antigen-uptake and activation. An explanation may thus be found for the advantages gained by using apoptotic and necrotic tumour cells to generate specific DC vaccines (Strome, Voss et al. 2002). In addition, characterization of the molecular mechanisms responsible for "cell death immunogenicity" may provide novel strategies to favour its occurrence during the preparation of killed tumour cell-based vaccines. The present results, indeed, showed that dying neoplastic cells, due to their impaired ability to expose CRT or HSP90, lose their immunogenic properties. Because BCL-2 can impair CRT surface translocation (Panaretakis, Kepp et al. 2009) and its up-regulation constitutes one of the hallmarks of indolent NHLs, in particular FL, the association between BCL-2 over-expression and the reduced tumour cell ability to expose CRT was investigated in 8 (4 Rs and 4 NRs) vaccinated patients. However, a precise correspondence between these two features was not found in these patients (data not shown) and the absence of available tumour cell samples for the remaining 10 patients hampered further investigations of the mechanisms that account for these deficiencies. As shown for murine colon carcinoma and sarcoma models (Obeid, Tesniere et al. 2007a; Obeid, Tesniere et al. 2007b), cytotoxic agents can be combined with adjuvant compounds such as recombinant CRT or PP1/GADD34 inhibitors to enhance CRT surface expression. Oligodeoxynucleotides (i.e., class A CpG), and cytokines (i.e., IL-1β) can also boost DC engulfing, cross-presentation, and maturation, and new ways of increasing the potency of DC-based vaccination may thus be devised (Zitvogel, Apetoh et al. 2008) (Locher, Rusakiewicz et al. 2009). This information combined with the results described

above lays the basis for improving the strategy of active immunotherapy using tumour cell-based vaccines.

4 Serological identification of NHL cell-surface antigen(s) using Ab repertoires of vaccinated patients

4.1 Introduction

Therapeutic mAbs alone or in combination with standard treatments have considerably improved the clinical management of several malignancies, including NHLs (Adams and Weiner 2005). In particular, the anti-CD20 mAb Rituximab in combination with conventional chemotherapy has significantly increased the survival of B-NHL patients and has become a standard choice for the treatment of these diseases (Cheson and Leonard 2008). However, it does not produce an effective therapeutic response in all patients (McLaughlin, Grillo-Lopez 1998; Cheson and Leonard 2008), and the majority of those that initially respond become "resistant" to repeated therapy (Davis TA Grillo-Lopez 2000).

Novel therapeutics with enhanced target-binding affinity or alternative, possibly tumour-restricted, specificity are thus required to improve the prognosis of NHL patients. Lymphoma-specific antigens, whose expression is not restricted to an individual lymphoma, have not been defined to date and few attempts have been made towards their identification (Huang, Preuss et al. 2002; Krackhardt, Witzens et al. 2002). One promising approach at this purpose is the study of the humoral immune response to TAAs. It is now largely established that cancer patients produce auto Abs to new epitopes derived from coding DNA mutations or genetic translocations in tumour cells (e.g., p53, BCR-ABL), or against proteins that show altered post-translational modifications (e.g., under-glycosylated MUC1) and expression in cancer, including over- (e.g., HER2/neu) and ectopic-expression (e.g., cancer testis antigens such as NY-ESO-1) (Sahin, Türeci, et al. 1995) (Houghton 1994). Thus, the identification of TAAs using cancer patients' circulating Abs appears a valuable strategy for the discovery of novel potential tumour biomarkers and targets for therapy (Huang, Preuss et al. 2002; Shoshan and Admon 2007).

The study of the humoral immune response to human tumours has been revolutionized since the introduction of the serological analysis of recombinant cDNA expression libraries (SEREX), exploiting patients' Abs to screen autoantigens encoded by tumour-extracted mRNA (Liggins, Guinn et al. 2005) (Sahin, Türeci, et al. 1995) (Krackhardt, Witzens et al. 2002). However, this approach carries certain technical drawbacks, these include the complexity and time-consuming nature of the SEREX process, and the lack of information on the overall range of the serospecificities in the individual cases and occurrence of particular serospecificities in patient populations (Desmetz, Cortijo et al. 2009). In addition, patient- but not cancer-related humoral responses are often detected as false positives, and antigenicity related to post-translational modifications cannot be identified due to the use of a prokaryotic system for recombinant protein expression (Desmetz, Cortijo et al. 2009). Serological proteome-based approach (SERPA), which combines the electrophoretic separation of cancer proteome and western blot analyses of patients' seroreactivities with mass spectrometry (MS) protein identification, has become a valuable and straightforward alternative. So far, SERPA has successfully characterized patients' humoral response against several types of cancer, including kidney (Klade, Voss et al. 2001), breast (Desmetz, Bascoul-Mollevi et al. 2009), colorectal (De Monte, Sanvito et al. 2008), pancreatic (Tomaino, Cappello et al. 2007) and ovarian cancer (Philip, Murthy et al. 2007), hepatocellular (Looi, Nakayasu et al. 2008) and squamous cell carcinoma (Fujita, Nakanishi et al. 2006; Yang, Xiao et al. 2007), as well as leukaemia (Cui, Li et al. 2005) and melanoma (Forgber, Gellrich et al. 2009). However, the clinical impact of these potential biomarkers has been limited, probably due to the need of a better understanding of the biological and clinical relevance of humoral immune responses against tumour antigens found in cancer patients (Desmetz, Bascoul-Mollevi et al. 2009).

The introduction of improved more sensitive MS devices and the development of high-throughput techniques based on synthetic protein microarrays and Ab chips has been generating a renewed enthusiasm about the possibility to successfully exploit the Ab repertoire of cancer patients for the identification of novel tumour-restricted diagnostic/prognostic biomarkers and/or biotargets for therapy (Caron, Choquet-Kastylevsky et al. 2007; Kijanka and Murphy 2009).

4.2 Aims of the Chapter

The major aim of this study was to identify novel potential therapeutic biotarget(s) of B-NHLs, exploiting the Ab repertoires of patients vaccinated with autologous DCs loaded with killed autologous tumour cells, as described in Chapter 2. In that pilot study, 6 out of 18 indolent B-cell lymphoma patients achieved objective clinical responses that positively correlated with anti-tumour immune-activation and with the occurrence of an immunogenic death in killed tumour cells used to prepare the patient-specific vaccines, as described in Chapter 3. Interestingly, only R showed the increase of circulating tumour-specific Abs after vaccination. These results led to the investigation as to whether the observed humoral responses were directed against common indolent NHL antigens, which could be exploited as novel targets for therapy. Towards this aim, tumour-specific cross-reactivity of Rs' post-vaccine Abs was initially verified. Then, allogeneic NHL antigens differentially recognized by vaccine-induced Abs in R were identified by SERPA, and their biological relevance was assessed studying their expression and functional role in normal and neoplastic cells.

4.3 Materials and Methods

4.3.1 Cell lines, primary cells, and culture conditions

The following human tumour cell lines were used: DOHH-2 and SC-1 (FL); RL-19, SU-DHL-6, KARPAS-422 and SU-DHL-4 (transformed DLBCL); GRANTA-519 (MCL); RAJI and NAMALWA (BL); HDMYZ and L-540 (HL); KMS-11 (multiple myeloma (MM)), MOLT-4 (Acute T Lymphocytic Leukaemia (ALL)) and SU-DHL-1 (Anaplastic Large T Cell Lymphoma (ALCL)). All cell lines were purchased from DSMZ (Braunschweig) and were routinely maintained in RPMI 1640 medium (Lonza) supplemented with 10% (v/v) fetal calf serum (Lonza) and L-glutamine (Lonza) in a humidified chamber (95% air, 5% CO2) at 37°C. Primary lymphoma cells were isolated from LN or PB as reported in Chapter 2. Written informed consent for the investigational use of lymphocytes, serum samples and tumour specimens was obtained from each patient in accordance with the Declaration of Helsinki.

4.3.2 IHC analysis

IHC was performed on tissue sections of indolent (n=54), aggressive NHLs (n=43) or non-malignant LNs (n=26) obtained from the Tissue Bank of *"Fondazione IRCCS Istituto Nazionale dei Tumori di Milano"*. Ab purification from human sera and their biotinylation were carried out as described in Chapter 2. Formalin-fixed and paraffin-embedded tissue sections, following epitope retrieval with 10 mmol/L of citrate buffer 0.07M pH 6.0 (Bioptica, Milan, Italy), were incubated with human biotinylated Igs (100 μg/ml) for 1 hour at RT and the immune reaction was developed using peroxidase-conjugated streptavidin. For Ki-67 and HSP105 staining, epitope retrieval was performed on formalin-fixed and paraffin-embedded tissue sections respectively treated with 6 mM citrate buffer for 6 min at 96°C or with 2 mM EDTA in distillated water pH 8 for 15 min at 98°C. Endogenous peroxidase activity was blocked by treatment with 30% hydrogen peroxide in PBS for 40 min and followed by treatment with normal goat serum (1:50) (Dako, Glostrup, Denmark) for 40 min at RT. Mouse anti-human Ki-67 mAb (1:100) (Ki-67 Antigen, Clone MIB-1, Dako) was incubated for 1 hour followed by biotin-conjugated anti-mouse IgG (1:100, Dako) for 1 hour at RT, whereas rabbit anti-human HSP105 polyclonal Ab (1:1500) (N-187, Santa Cruz Biotecnology, CA, USA) was incubated overnight at 4°C followed by biotin-conjugated anti-rabbit IgG (1:200, Dako) for 1 hour at RT. The immunoreactivity was then revealed by HRP-conjugated streptavidin (1:300, Dako). The presence of immune-conjugated was visualized using the chromogenic substrate 3,3'-diaminobenzidine (Sigma) and counterstaining with hematoxylin. Ki-67 expression was scored as weak (5-10%), moderate (10-30%) and strong (>30%) based on the number of positive nuclei in tumour cells. HSP105 combining score (CS) was evaluated as low (0-2), moderate (3-5) and high (6-7) based on the % of positive neoplastic cells (0-4; 0= ≤1%, 1= >1% and ≤20%, 2= >20% and ≤50%, 3= >50% and ≤80%, 4= >80%) and staining intensity (1-3; 1= weak, 2= moderate, 3= strong). Sections were analysed by acquiring digital images of each tissue section (X10, X20 and X40) using Leica DMD108 (Heidelberg, Germany) equipped with HI Plan 10X/0.25 and 40X/0.65 objectives and processed using Adobe Photoshop CS4 software (San Jose, CA).

4.3.3 Flow cytometry

Surface staining was performed on live tumour cells, as described in the previous Chapters, after blocking nonspecific Ab binding to the Fc-receptors (FcR blocking reagent, Miltenyi Biotec). Cytoplasmic staining was carried out after fixation and permeabilization with BD Cytofix/CytopermTM (BD Pharmingen, San Diego, CA), according to the manufacturer's instructions. Immunoreactivity of biotin-conjugated human serum-derived

polyclonal Igs (100 μg/ml) and rabbit anti-human HSP105 polyclonal Ab (4 μg/ml, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) were revealed respectively by FITC-labelled streptavidin (1:80) (BD Pharmingen) and FITC-labelled goat anti-rabbit Igs (Jackson Imunoresearch). As negative controls, cells were incubated in parallel with the matched Ig isotype (Santa Cruz Biotechnology, Inc.). Samples were acquired by setting photomultiplier tubes on the autofluorescence of the related negative control.

4.3.4 MTT assay

NHL cell lines were seeded in round-bottomed 96-well plates (10.000 cells/200 μl /well) in the presence of patients' post-vaccine Igs (1:50), Igs purified from donors' serum (1:50), rabbit anti-human HSP105 polyclonal Ab (two-fold dilution, range 8-0.125 μg/ml, Santa Cruz), or the matched Ig isotype (Santa Cruz) at the same concentrations as control. After 72-hour culture, 20μl of 5mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) was added directly to the wells. Following 4-hour incubation in a humidified chamber (95% air, 5% CO2) at 37°C, plates were centrifuged for 10' at 2000 rpm and the supernatant was removed from each well. The converted formazan dye was then solubilised in DMSO (100μL/well). Absorbance was read at a wavelength of 550 nm with an automated ELISA microplate reader (Bio-Rad).

4.3.5 OFFGEL fractionation

Protein isoelectro-focusing was performed by *the Proteomics Unit* at our Institute. To this aim, the 3100 OFFGEL Fractionator and the OFFGEL Kit 3–10 (Agilent Technologies, Milan, Italy) were used following manufacturer's instructions. The device was set up for the 24 fractions separation by using 24-cm-long immobilized pH gradient (IPG) gel strips with a linear pH gradient (range 3–10). DOHH-2 cell proteins were separated in a two-phase system: liquid upper phase separated in wells, and lower IPG gel strip phase. The sample was focused using the recommended method for OFFGEL proteins 24 wells fractionation with a maximum current of 50 μA. Separation method consisted in cooling platform temperature at 15°C; with electrical setting parameters: 8000V/h, 100000 V, 200W and 50μA/strip. The focusing was stopped after total voltage reached 64 kVh. After focusing, samples were recovered from each well and transferred in individual micro tubes. Corresponding protein fractions were purified with 2-D Clean Up Kit and protein pellet dissolved in running sample buffer compatible with 1-D SDS-PAGE.

4.3.6 Western blotting and Immunoprecipitation

Lymphoma cell lines were solubilised for 40 min at 0°C with lysis buffer as described in Chapter 3. Glycoproteins were purified from total cell lysates as described (Pathak et al.) using the Qproteome Total Glycoprotein Kit (Qiagen). Briefly, the cleared lysates were applied to equilibrated affinity columns containing concanavalin A, which binds mannose, and wheat germ agglutinin, which binds sialic acid, and bound glycoproteins were eluted 3 times with elution buffer containing mannose, followed by elution 3 times with elution buffer containing sialic acid, each buffer supplemented with protease inhibitor solution (100x) and the detergent solution (10x) according to the the kit instructions. After pooling the eluates, glycoprotein sample concentration were determined with Bradford reagent (Biorad).

For immunoprecipitation, lysates were precleared for 30 min at 4°C with immunopure immobilized protein A/G sepharose (Pierce) previously equilibrated in lysis buffer. Precleared lysates (3 mg DOHH-2 protein lysate/sample) were incubated for 2 h at 4°C with 3μg anti-HSP105 Ab or rabbit normal serum as negative control. They were subsequently incubated with aliquots of protein A/G-Sepharose (60 μl/sample, previously

equilibrated in lysis buffer) overnight at 4°C on a rocker. Sepharose bound-immune complexes were washed three times with lysis buffer, eluted in loading buffer and divided in three aliquots to be successively separated in 1-D SDS-PAGE.

Eluted immunoprecipitated proteins, or OFFGEL protein fractions or lymphoma cell lysates, mixed with loading buffer and denatured upon heating for 5 min at 95 °C, were subjected to electrophoresis on 4-12% or 3-8% NuPAGE® Bis-Tris precast polyacrylamide gels (Invitrogen, Carlsbad, CA, USA). Separated proteins were transferred onto nitrocellulose membrane (Hybond-C Super; Amersham Biosciences, GE Healthcare) in 20% ethanol NuPAGE® transfer buffer (Invitrogen), stained with red ponceau solution, washed extensively with 0.5% Triton X-100 PBS and saturated overnight at 4°C in blocking solution (5% low-fat milk, 0.1% Triton X-100 PBS) before incubation with primary Abs (biotin-conjugated Igs purified from patients' pre- and post-vaccine serum samples (100 µg/ml), rabbit anti-human HSP105 polyclonal Ab (1µg/ml, Santa Cruz), or rabbit anti-human actin polyclonal Ab (1:1000, Sigma, Milan, Italy)) in blocking solution for 1 hour at RT. Immunoreactive proteins were visualized using appropriate secondary antibodies: HRP-conjugated donkey anti-rabbit Ig (1:10,000) (Amersham Biosciences, GE Healthcare) or HRP-conjugated streptavidin (1:10,000) (Amersham Biosciences, GE Healthcare), and signals were detected using an enhanced ECL system (ECL Western Blotting Detection Reagents; GE Healthcare) according to the manufacturer's protocol.

4.3.7 In-gel tryptic digestion, MALDI-TOF-MS analysis and peptide mass fingerprinting

For protein identification, protein bands were excised from silver-stained preparative gel and processed as described in Chapter 3 (Paragraph 3.3.7).

4.3.8 In-vivo experiments

Severe combined immunodeficient (SCID) mice (6-8 weeks old) with body weight of 20 to 25 g were purchased from Charles River (Calco, Italy).

Mice were injected subcutaneously with $10x10^6$ NAMALWA (N=54) and SU-DHL-4 (N=36) cells. When tumours reached 0.05-0.07 cm^3, mice were randomized in three groups (n=6/group, for a total of three and two different experiments with NAMALWA and SU-DHL-4 models, respectively) to receive 3 intraperitoneal injections of 1 mg/ml rabbit anti-human HSP105 polyclonal Ab or matched rabbit Ig isotype (250 µg/250 µl mouse, azide-free preparation for in vivo use, Santa Cruz Biotechnology, Inc.) or NaCl solution 0.9% (250 µl/mouse). Tumours were calibrated twice/three times a week and tumour volume was calculated as $0.5 \times d1^2 \times d2$, where d1 and d2 are the smaller and larger diameters, respectively.

In-vivo tumour growth was also evaluated (n=3 mice/group) by magnetic nuclear resonance (MNR) 24 hours after the second treatment, when tumours reached the maximum growth inhibition. MNR imaging was performed at *the Radiology Unit* of our Institute with 1.5-T systems (Achieva; Philips, The Netherland) by using similar pulse sequences. In all cases, coronal short tau inversion recovery (STIR) sequences, axial turbo-spin-echo (TSE) T2-weighted sequences and axial and coronal unenhanced TSE T1-weighted sequences were followed by an axial and coronal contrast-enhanced TSE T1-weighted sequences (section thickness 2 mm).

After 24 hours from the last treatment, tumours were excised and processed for IHC. Paraffin-embedded biopsies from NAMALWA and SU-DHL-4 xenografts were analyzed for HSP105 and Ki-67 expression as described in Paragraph 4.3.2. CD45, CD56 and granzyme-B immunostainings were performed on tissue sections from tumour xenografts using the following primary Abs: mouse anti-human CD45 (clones RP2/18 and RP2/22, Novocastra, UK), rat anti-mouse CD56 (clone H28.123, Abcam, USA), rat anti-mouse granzyme-B (clone

16g6, eBioscience). Binding of primary Abs was revealed by using the streptavidin-biotin-peroxidase complex method and 3-3'-diaminobenzidine as chromogenic substrate.

In-vivo staining of tumour vasculature with sulfosuccinimidyl-6- (biotinamido) hexanoate-biotin (Thermo Fisher Scientific) was performed after 24 hours from the last treatment to evaluate tumour vasculature injury, as described (Lavazza, Carlo-Stella et al. 2010). Experimental protocols were approved by the Ethical Committee for Animal Experimentation of the *Fondazione IRCCS Istituto Nazionale dei Tumori di Milano*, according to the Italian laws (D.L. 116/92 and after additions), which enforce the EU 86/109 Directive.

4.3.9 Statistical analysis.

Two-sided Student's "*t*" test ($p \leq 0.05$) for paired or unpaired data was used to test statistically significant differences. The non-parametric test Spearman rank correlation was used to measure the degree of association between two variables. Tumour volume data from the *in-vivo* study were analysed with 2-way analysis of variance (ANOVA), and individual group comparisons were evaluated by Bonferroni posttest. Statistical analyses were performed on the Prism 5.0a software version for Macintosh Pro personal computer (GraphPad Software, Inc.).

4.4 Results

4.4.1 DC-based vaccination induced the development of cross-reactive therapeutic Abs

In Chapter 2, it was already shown that DC-based vaccination was able to induce a humoral immunity against the autologous tumours in clinical Rs. In order to analyse whether vaccine-induced anti-tumour Ab response was directed to shared lymphoma-restricted antigens, total Igs isolated from patients' serum samples collected before and after vaccination were biotinylated and tested on allogeneic tumour biopsies. IHC analysis revealed tumour-restricted cross-reactivity only when post-vaccine Igs from Rs, either CR or PR, were used (Figure 4.1Ai, Aii right); whereas pre-vaccine Igs from the same Rs or pre- and post-vaccine Igs from NRs showed no tumour-restricted immunostaining (Fig. 4.1Ai, Aii left and Aiii left and right). To study the cellular localization of the target TAAs, biotin-conjugated patients' Igs were tested by flow cytometry on live NHL cells, either as cell lines or from primary tumours, and normal B and T cells (Figure 4.1B). Vaccine-induced Ab responses to cell-surface tumour-specific antigens, evaluated as the ratio between post- and pre-vaccination Ig reactivity, were significantly higher in Rs compared to NRs (Figure 4.1B for representative examples). As shown in Figure 4.1B (top), the ability of post-vaccination Igs from CR to recognize live tumour cell lines and primary cells increased by an average of 18-fold and 8-fold respectively compared to normal B cells. By contrast, post-vaccination Igs from NR did not react with significantly enhanced efficiency on live tumour versus normal B cells (Figure 4.1B bottom).

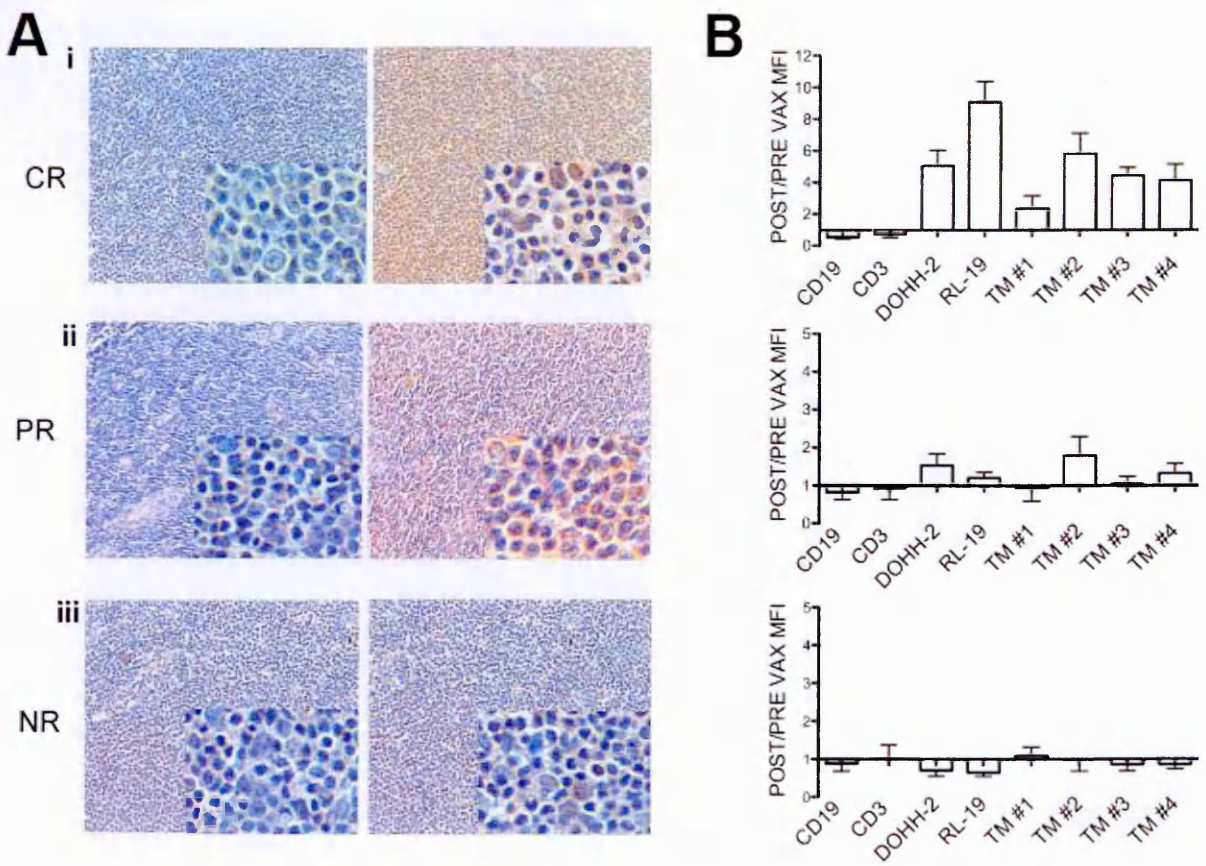


Figure 4.1 Tumour-restricted patients' Igs cross-reactivity.
(A) Representative IHC analysis showing immunoreactivity of biotin-conjugated pre- (left) and post-vaccine (right) Igs of one CR (top), one PR (middle) and one NR (bottom) tested on allogeneic human NHL specimens

using HRP-streptavidin revealing system. Panels and their insets are shown, respectively, at X10 and X40 magnification. (B) Flow cytometry analysis of the same Ig samples used in A (CR top, PR middle, NR bottom) represented as the ratio between post- and pre-vaccine Ig MFI revealed by FITC-labelled streptavidin on normal B (CD19) and T (CD3) cells, NHL cell lines (DOHH-2 and RL-19) and 4 different primary FL cells (TM #1-4).

These findings suggested that the induction of Abs directed against lymphoma cell surface antigens was associated with a clinical response to vaccination.

To study the therapeutic potential of Abs induced in CRs after vaccination, DOHH-2 cells were cultured in the presence of donors' Igs or post-vaccine Igs obtained from all the 3 CRs of the study and 2 NRs, and their growth was measured after 72 hours in a standard MTT assay (Figure 4.2).

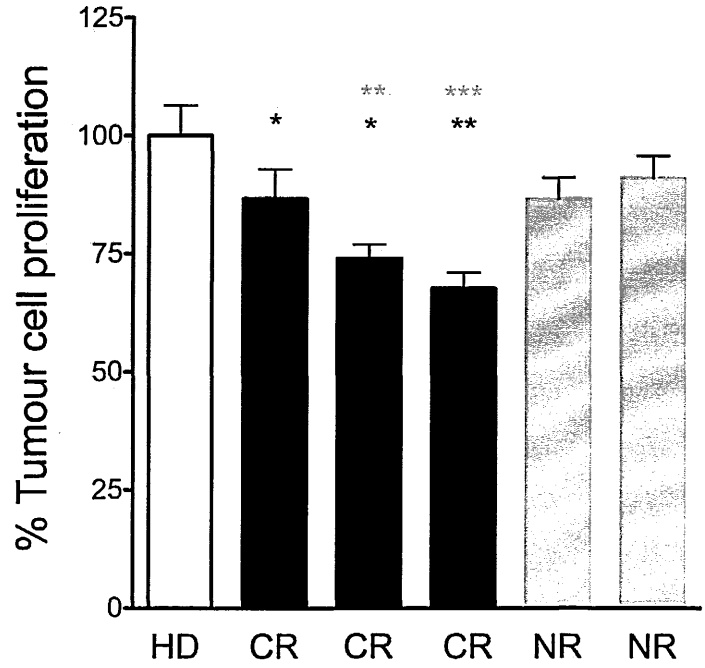


Figure 4.2 In-vitro anti-tumour activity of purified post-vaccine Igs of CRs.
Igs purified from pooled healthy donor serum samples (HD, white) or from post-vaccine serum of 3 CRs (black columns) or 2 NRs (grey columns) were incubated with DOHH-2 cells for 72 hours in a standard MTT assay. Data are mean and standard deviation of 4 replicates. Significance was calculated using unpaired Student's *t* test (*=$p \leq 0.05$; **=$p \leq 0.01$; *** = $p \leq 0.001$, black stars: black vs. white columns; grey stars: black vs. grey columns).

Interestingly, Abs contained in post-vaccine serum of all CRs significantly impaired DOHH-2 cell growth compared to donors' Igs and either of the NRs' samples (Figure 4.2; $p=0.05$, $p=0.01$, $p=0.004$ and $p=0.01$, $p=0.001$), suggesting that vaccination induced the production of tumour-specific Abs that might have contributed to the observed clinical efficacy.

The findings above provide evidence for the existence of NHL widespread immunodominant antigen(s) that could be exploited as biotargets for a novel successful therapy.

4.4.2 Serological identification of HSP105 as a novel B-cell lymphoma antigen

To identify the lymphoma-specific antigen(s) therapeutically targeted by Rs' post-vaccine Igs, biotin-conjugated patients' pre- and post-vaccine Igs were tested on DOHH-2 total cell lysate by western blot. This preliminary approach revealed a sharp protein band

migrating at about 110 kDa with Rs' post-vaccine sample (Figure 4.3 Ai, lanes 2), but not with their pre-vaccine Igs (Figure 4.3 Ai, lanes 1) or with NRs' pre- and post-vaccine Igs (Figure 4.3 Aii, lanes 1 and 2). The same strategy, applied to DOHH-2-purified glycoproteins, identified a differential protein band migrating at a similar molecular weight with Rs' post-vaccine Igs amongst sialylated (Figure 4.3B, right, lane 2 vs. lane 1) but not mannosylated proteins (Figure 4.3B, left). These findings indicated that vaccination induced the production of Abs directed against lymphoma-associated glycoproteins, which, being preferentially expressed on the cell-surface, might represent very attractive targets for mAb therapy.

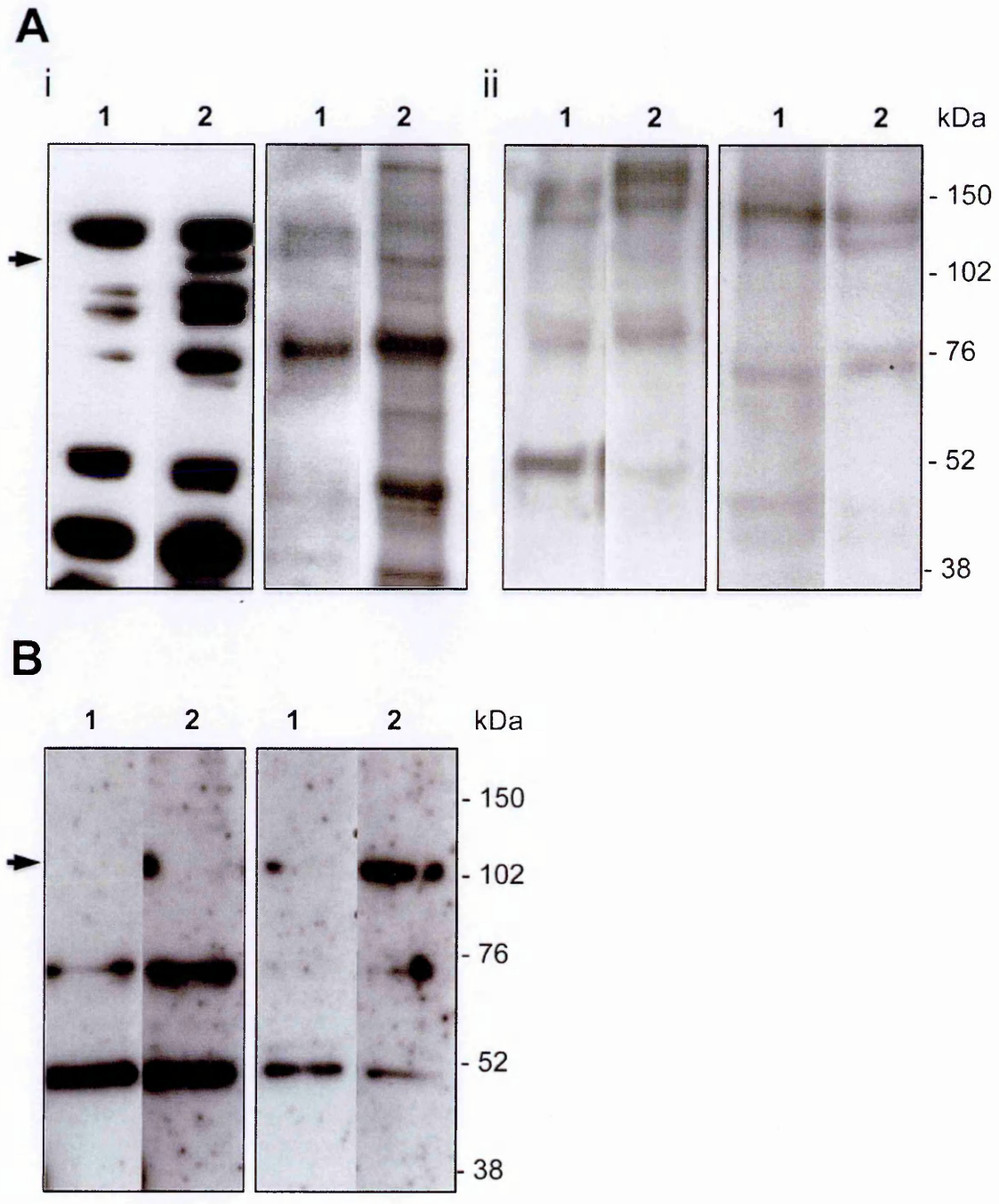


Figure 4.3 Western blot analyses of biotin-conjugated patients' Igs.
Western blot analyses of DOHH-2 cell extracts probed with biotin-conjugated Igs isolated from pre- (lanes 1) and post- (lanes 2) vaccine serum samples of Rs (i) and NRs (ii); (B) western blot analyses of DOHH-2 purified mannosylated (left) and sialylated (right) proteins probed with pre- (lanes 1) and post-vaccine (lanes 2) Igs from the same R serum samples shown in left panel of Figure Ai.

To identify the differentially recognized proteins migrating at about 110kDa, a 2-step SERPA was applied. Initially, DOHH-2 glycoproteins were divided into 24 fractions according to their isoelectric point (p.I.), and each fraction was analysed by standard western blot using the same Ig samples showed in left panel of Figure 4.3Ai. The comparison of pre- and post-vaccine Ig reactivity for all the 24 fractions revealed 2 differential bands migrating at about 110kDa in the low pH fractions only when the post-vaccine sample was used (Figure 4.4B, left panel, red arrows). In addition, new or more intense bands were detected with post- compared to pre-vaccine Igs, further indicating that vaccination boosted the production of Abs directed against other lymphoma-associated glycoproteins (Figure 4.4B, black arrows).

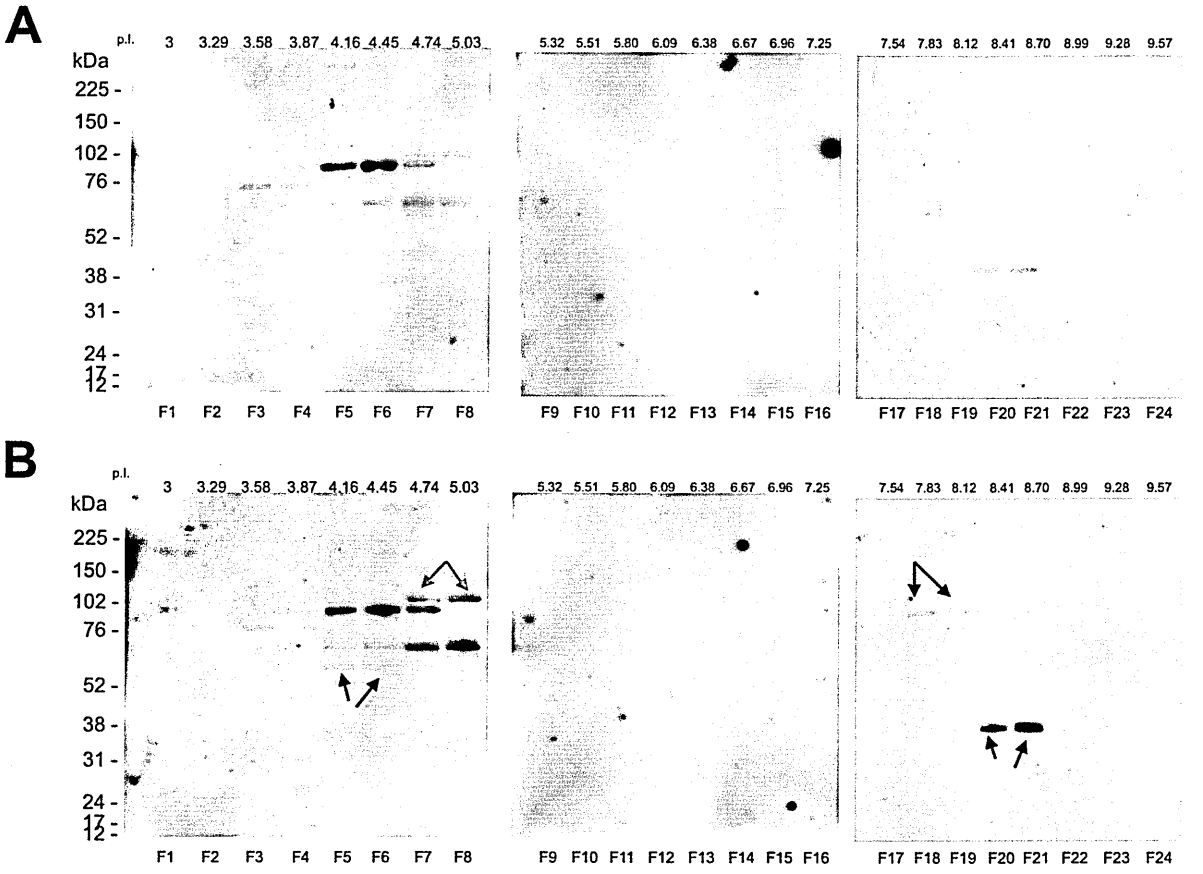


Figure 4.4 Western blot analyses of OFFGEL fractionated DOHH-2 glycoproteins.
Western blot analyses of DOHH-2 glycoproteins separated according to their isoelettric point (p.I.) into 24 fractions (F1-24) and probed with R's pre- (A) and post-vaccine (B) serum-derived Igs used in the left panel of Figure 4.3Ai. Arrows indicate differentially reacting bands.

The same analysis conducted on the whole lysate of DOHH-2 cells revealed additional proteins compared to those illustrated in Figure 4.4, and consistently showed one differential band migrating at about 110kDa in fraction "F6" only when post-vaccine Igs obtained from this R were used (Figure 4.5A vs. B, red arrow).

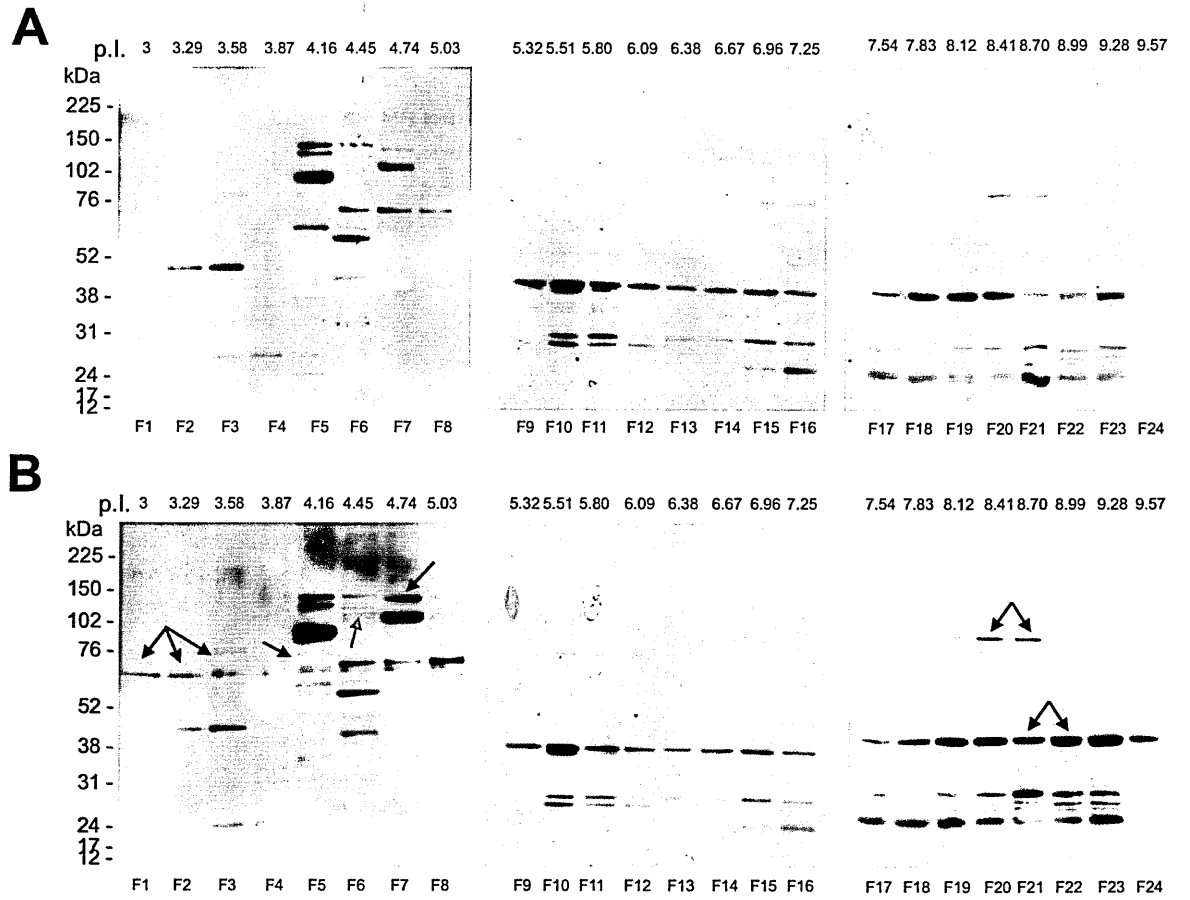


Figure 4.5 Western blot analyses of OFFGEL-fractionated DOHH-2 total proteins.
Western blot analyses of 24 protein fractions (F1-24) separated from DOHH-2 cell whole lysate according to their isoelettric point (p.I.) and probed with R's pre- (A) and post-vaccine (B) serum-derived Igs used also in Figure 4.4. Arrows indicate differentially reacting bands.

MALDI-TOF-MS analysis, performed in that molecular weight range of a silver-stained preparative gel, identified in F6, and not in the non-reactive adjacent F5 and F7, U5S1 (component of the U5 small nuclear ribonucleoprotein particle), puromycin-sensitive aminopeptidase (PSA), mini-chromosome maintenance proteins (MCM)-4 (a DNA helicase activity factor), all constitutively expressed in the nucleus or cytoplasm, and HSP105, as the potential proteins differentially recognized by post-vaccination Igs (Figure 4.6A). In light of the possibility that HSP105 could be expressed on cell surface and the widely described roles of HSP family members in a broad range of neoplastic processes (Whitesell and Lindquist 2005; Luo, Solimini et al. 2009) the study was focused on understanding HSP105's involvement in B-NHLs.

Western blot analysis of DOHH-2 glycoprotein (F6-8, Figure 4.6B) and whole cell lysate (F5-7, Figure 4.6C) fractions probed with a commercial anti-HSP105 Ab revealed the presence of HSP105 specific band in the same fractions (glycoprotein F7 and F8 fractions and the whole lysate F6 fraction) and at a comparable molecular weight to that recognized by the Rs' post-vaccine Igs, thus validating the results obtained by SERPA.

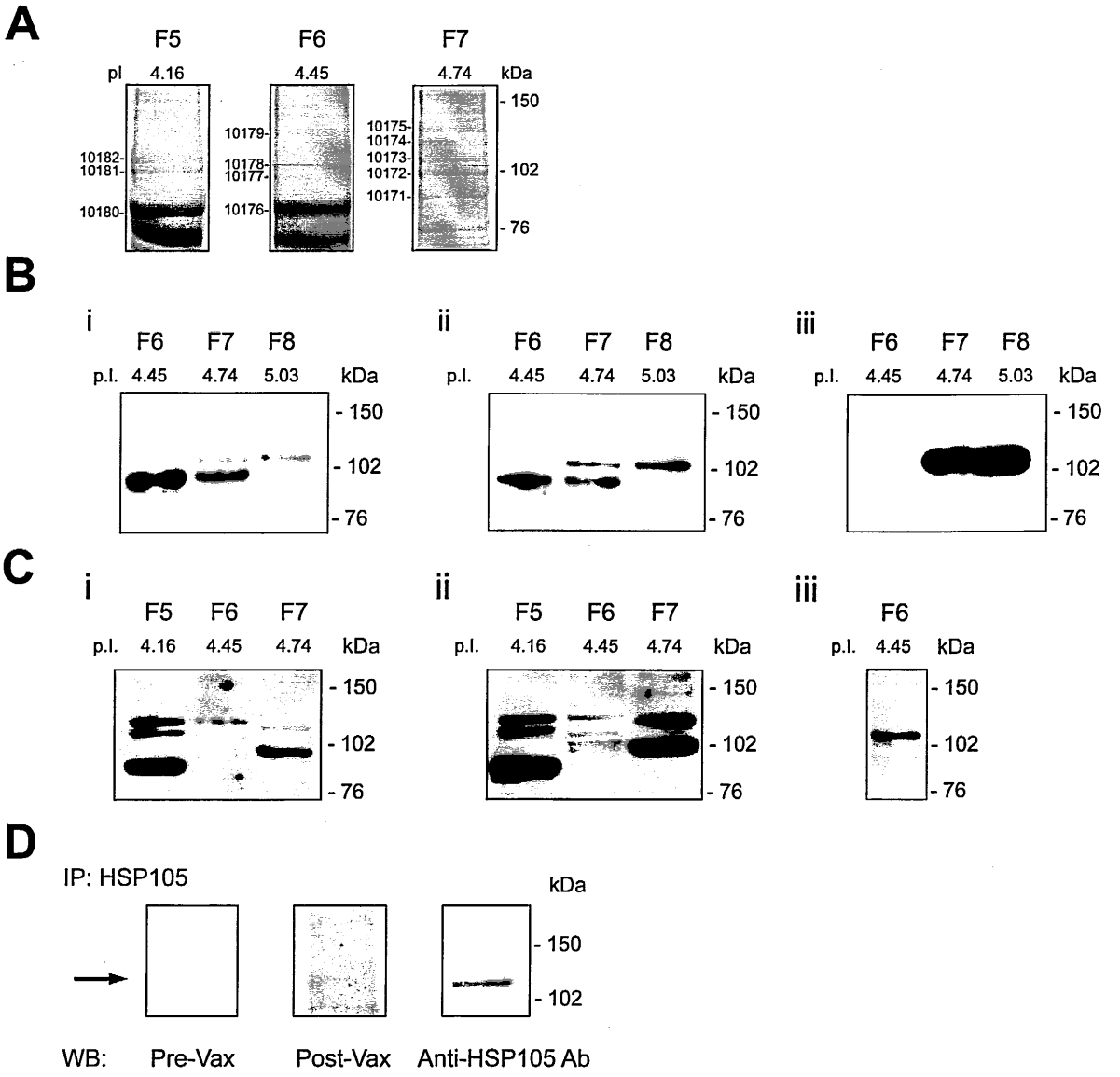


Figure 4.6 MALDI-TOF-MS-based identification of HSP105 in B-NHL.
(A) Silver staining of F5, F6 and F7 preparative SDS-PAGE for MS analysis of the numbered protein bands. (B) Comparison of DOHH-2 glycoprotein F6-8 fractions probed with R's pre- (i), post- (ii) vaccine Igs or (iii) commercial anti-human HSP105 Ab. (C) Comparison of DOHH-2 total cell lysate protein F5-7 fractions probed with R's pre- (i), post- (ii) vaccine Igs or (iii) commercial anti-human HSP105. (D) Immunoprecipitation of HSP105 from total DOHH-2 cell lysate with a commercial anti-human HSP105 Ab, followed by western blotting analysis with R's pre- and post-vaccine Igs, or anti-HSP105 Ab, as positive control. IP: immunoprecipitation; WB: Western blot.

In addition, HSP105 was immunoprecipitated from DOHH-2 total cell lysate with a commercial anti-HSP105 Ab, and then probed with R' Ig samples used in SERPA. HSP105 was better revealed by post-vaccine Igs rather than by the matched pre-vaccine sample (Figure 4.6D), indicating the increase of circulating anti-HSP105 Abs in this R after vaccination.

4.4.3 Expression analysis of HSP105 in NHL cell lines and primary tumours

HSP105 expression was analysed by western blot in a panel of tumour cell lines of B-(Figure 4.7, lanes 2-13) and T-cell origin (Figure 4.7, lanes 15-16) and their normal cell counterparts (Figure 4.7, lanes 1 and 14, respectively). As shown in Figure 4.7, the α-

isoform, which is the one migrating at the highest molecular weight, was found expressed in all cells analysed, even though to a lesser extent in normal B and T cells (lanes 1 and 14). By contrast, the inducible β-isoform was preferentially expressed by neoplastic cells, although at variable levels (Figure 4.7).

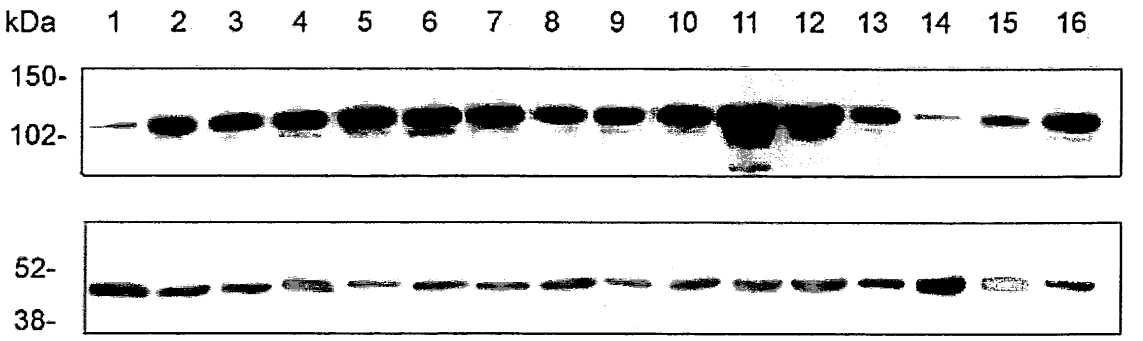


Figure 4.7 Western blot analysis of HSP105 expression in normal and neoplastic B and T cells.
One-dimensional SDS-PAGE of proteins extracts from normal B cells (1), B-NHL cell lines DOHH-2 (2), SC-1 (3), SU-DHL-6 (4), KARPAS-422 (5), RL-19 (6), SU-DHL-4 (7), RAJI (8), NAMALWA (9) and GRANTA-519 (10), Hodgkin lymphoma cell lines L-540 (11) and HDMYZ (12), multiple myeloma cell line KMS-11 (13), normal T cells (14), T cell leukaemia cell lines MOLT-4 (15) and T-cell lymphoma SU-DHL-1 (16) probed with commercial rabbit anti-human HSP105 polyclonal Ab (top) and rabbit anti-human Actin polyclonal Ab (bottom).

To analyse the subcellular localization of HSP105, its expression was studied by flow cytometry on the same cells, used either alive (Figure 4.8A), or upon being fixed and permeabilized (Figure 4.8B). Interestingly, B-NHL cell lines show a gradual increase of HSP105 surface-expression according to their aggressiveness, when both the MFI (Figure 4.8A left) and positive cell percentage (Figure 4.8A right) were analysed. Donor T cells (HD CD3) or cell lines derived from T-cell leukaemia and lymphoma did not express surface-HSP105 (Figure 4.8A). HSP105 was expressed on the surface of about 50% of normal circulating B cells (Figure 4.8A right), suggesting that the exposure of HSP105 may have a role in B-cell physiology and lymphomagenesis. As expected, HSP105 was expressed within the cytoplasm of all the cell lines tested (Figure 4.8B). However, intracellular staining was heterogeneous in B-cell malignancies (Figure 4.8B, columns 2-13), whereas it was comparably high in normal and neoplastic T cells (Figure 4.8B, columns 14-16).

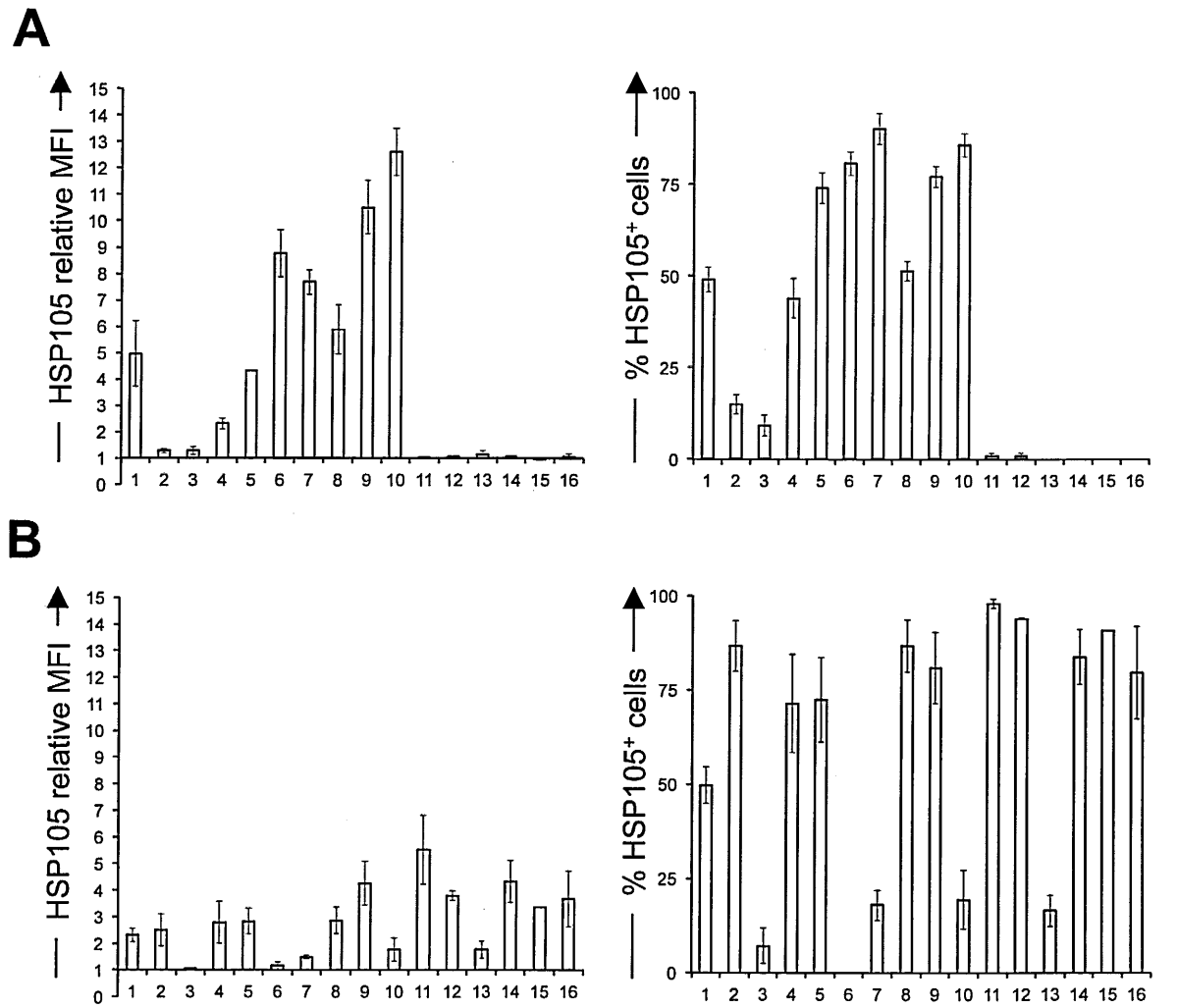


Figure 4.8 FACS analysis of HSP105 expression in normal and neoplastic B and T cells.
Flow cytometry analyses of surface (A) and intracellular (B) staining of anti-HSP105 Ab in normal B cells (1), B-NHL cell lines DOHH-2 (2), SC-1 (3), SU-DHL-6 (4), KARPAS-422 (5), RL-19 (6), SU-DHL-4 (7), RAJI (8), NAMALWA (9) and GRANTA-519 (10), Hodgkin lymphoma cell lines L-540 (11) and HDMYZ (12), multiple myeloma cell line KMS-11 (13), normal T cells (14), T cell leukaemia cell lines MOLT-4 (15) and T-cell lymphoma. Relative MFI was calculated as ratio between stained sample and negative control MFI.

The expression of HSP105 was then evaluated in primary NHL biopsies and normal LNs by IHC. In order to achieve this, a CS, ranging from 0 to 7, was arbitrarly defined to quantify the levels of HSP105 expression based on both the positive cell percentage and staining intensity, as described in the Materials and Methods section of this Chapter. To ascertain whether the presence of the antigen was associated with the clinical response to vaccination, the extent of HSP105 expression in the tumours of Rs were initially compared with that detected in tumours of NRs. The analysis of the available tumour biopsies (10 out of 18) revealed no significant differences in HSP105 expression level according to the clinical outcome of the patients, indicating that the lack of response was not due to a reduced lymphoma expression of HSP105 (Figure 4.9).

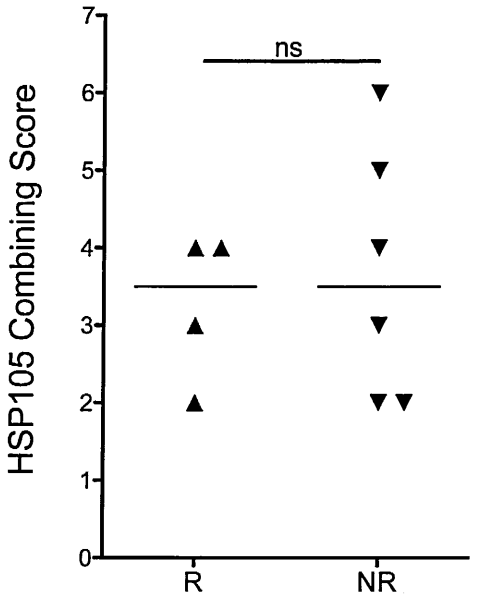


Figure 4.9 Immunohistochemical analysis of HSP105 expression in tumour biopsies from vaccinated patients.
Combining score for HSP105 expression in tumour biopsies from 4 Rs and 6 NRs. Analysis of statistical significance was performed with Student's *t* test (ns: not significant).

The IHC analysis was thus widened to include additional cases of both indolent (n=54) and aggressive (n=43) NHLs, as well as non-malignant LNs (n=26) biopsies. In this case, HSP105 expression was analysed in relationship to disease aggressiveness and malignancy, evaluated on the basis of lymphoma histological grade and Ki-67 staining, respectively. A CS=4 was arbitrarily considered as a threshold value that mainly discriminated between the low- and the high-grade B-NHLs (Figure 4.10). HSP105 expression was significantly higher in the aggressive compared to the indolent B-NHLs or normal LNs (Figure 4.10A, $p<0.001$) and was directly correlated with the lymphoma histological grade (Figure 4.10A, $p<0.0001$, Spearman $r=0.6951$). In addition, high-proliferating tumors, characterized by an intense and widespread Ki-67+ staining (>50% Ki-67+ nuclei), expressed HSP105 at significantly higher levels compared to the low-proliferating forms (<50% Ki-67+ nuclei) (Figure 4.10B, $p<0.001$). The lymphoma proliferation rate thus correlated with HSP105 expression levels (Figure 4.10B, $p<0.0001$, Spearman $r=0.7473$).

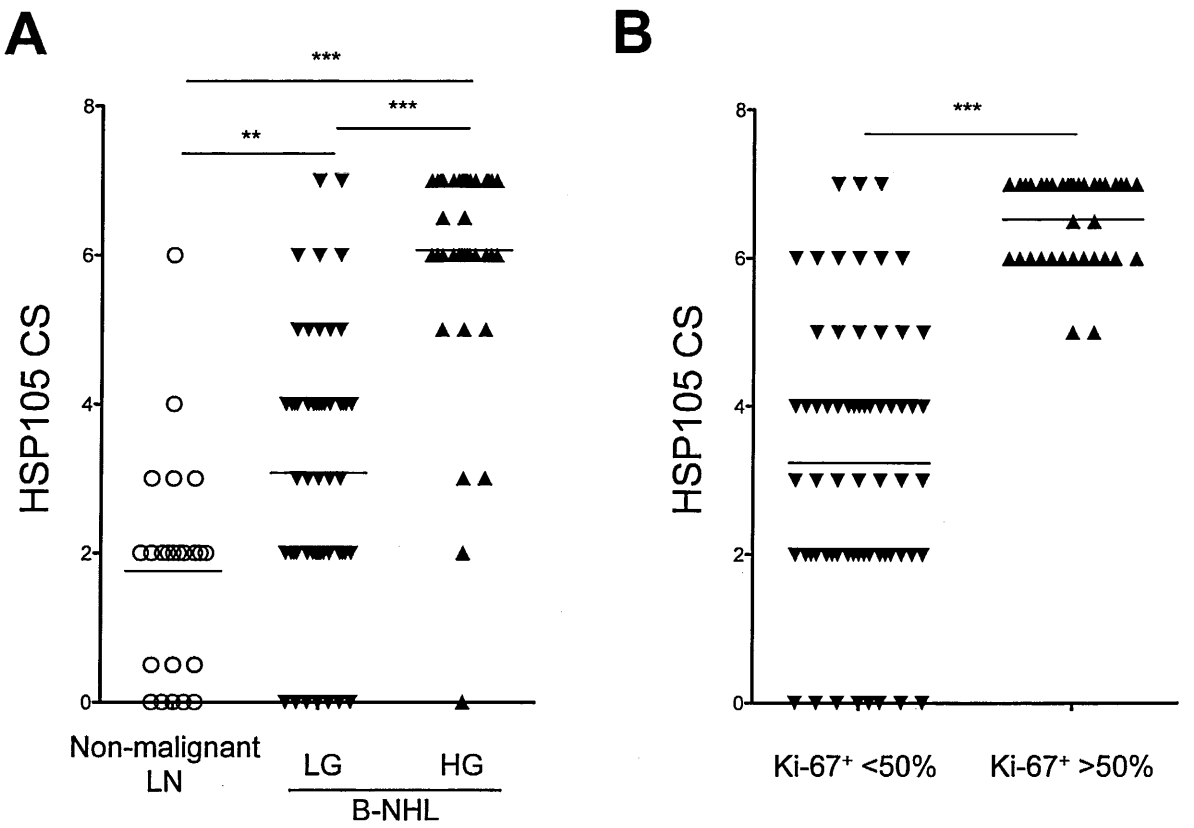


Figure 4.10 Immunohistochemical analysis of HSP105 expression in normal and neoplastic LNs. Combining score for HSP105 expression in NHLs and normal LNs plotted against tumour aggressiveness based on lymphoma histological grade (A) or Ki-67 expression level (B). Analysis of statistical significance was performed with Student's *t* test (*: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$).

Even in normal reactive LNs and indolent NHLs, where the structure of the follicles was preserved, HSP105 was preferentially expressed in the GC lymphocyte-enriched areas (Figure 4.11A right, for a representative example) that also stained strongly for Ki-67 (Figure 4.11A middle, for a representative example), suggesting a potential involvement of HSP105 in cell-cycle regulation of normal or malignant B cells. Further support for this was provided by the study of a case of indolent NHL before and after its progression. Increased HSP105 expression coupled with a stronger Ki-67 staining was observed in the progressed DLBCL (Figure 4.11Bii) compared to the diagnostic low-grade lymphoplasmacytic lymphoma (LPL) (Figure 4.11Bi).

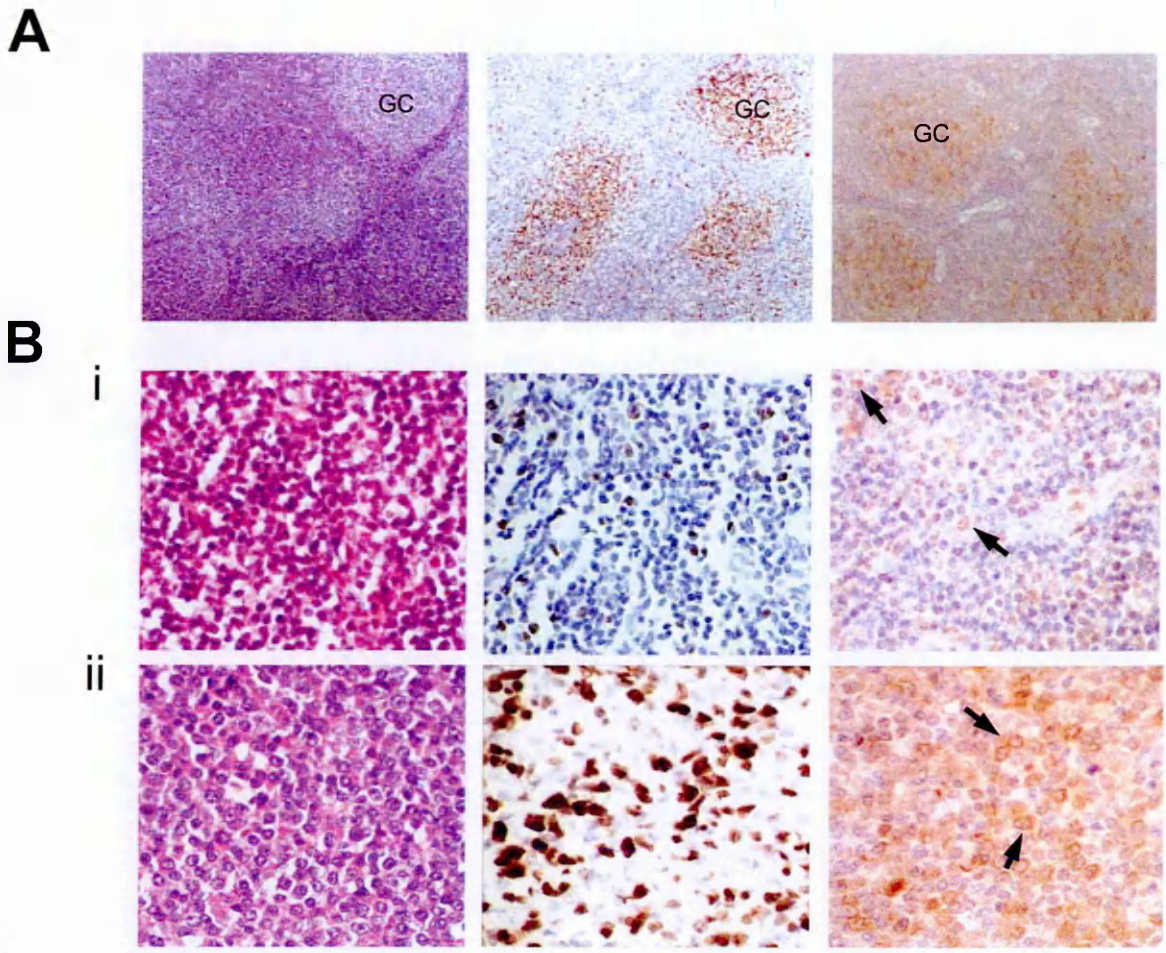


Figure 4.11 Example of immunohistochemical analysis of HSP105 and Ki-67 in low- and high-grade B-NHLs.
Hematoxylin eosin (left), Hi-67 (middle) and HSP105 (right) staining in one FL (A) and one LPL specimen (B) before (i) and after progression (ii) towards DLBCL. Arrows indicate HSP105 expression on lymphoma cell surface. Panels in A and B are shown, respectively, at X10 and X20 original magnification. GC: germinal centre.

4.4.4 In-vitro and in-vivo anti-lymphoma effects of HSP105 targeting

The therapeutic potential of targeting HSP105 with a specific Ab was then investigated by standard MTT assays using different B-NHL cell lines. The concentrations of anti-HSP105 Ab or matched control isotype Igs that achieved a 50% growth inhibition (IC50) in culture after 72 hours of incubation are showed in Figure 4.12. Amongst the cell lines tested, SU-DHL-4 and NAMALWA resulted the most sensitive to the specific treatment compared to the control isotype Igs (Figure 4.12, p=0.02, p=0.04).

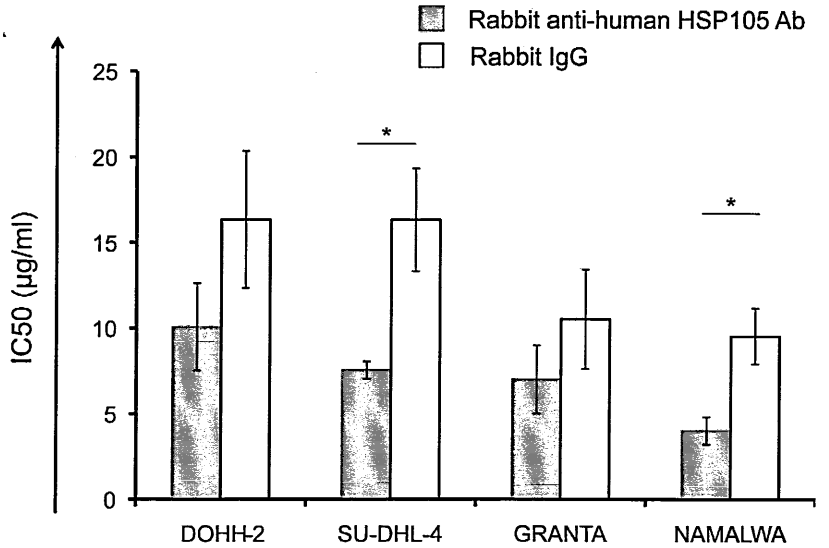


Figure 4.12 In-vitro anti-tumour effects of anti-HSP105 Ab against lymphoma cell lines.
Rabbit polyclonal anti-HSP105 Ab (black column) or matched isotype Igs (white column) concentrations reaching 50% growth inhibition (IC50) of DOHH-2, SU-DHL-4, NAMALWA and GRANTA-519 cell line cultures after 72 hour-incubation, measured using standard MTT assays. The average values of 3 independent experiments are shown. Analysis of statistical significance was performed with Student's *t* test (*: $p \leq 0.05$).

In the attempt to define the potential therapeutic role of HSP105 targeting in B-cell lymphomas, anti-HSP105 Ab was tested in SCID mice xenografted with these cell lines. As control groups, mice were treated in parallel with the matched isotype Igs or NaCl solution. The *in-vivo* targeting of HSP105 was associated with a significant reduction of tumour burden in both NAMALWA and SU-DHL-4 models with respect to the control treatments, as measured by calliper (Figure 4.13A and B, respectively) or MNR in a limited number of cases (Figure 4.13C and D, respectively). After only two administrations of anti-HSP105 Ab, the NAMALWA and SU-DHL-4 tumours exhibited an average growth inhibition of 70% and 60%, respectively, compared to those present in the untreated animals (Figure 4.13A and B).

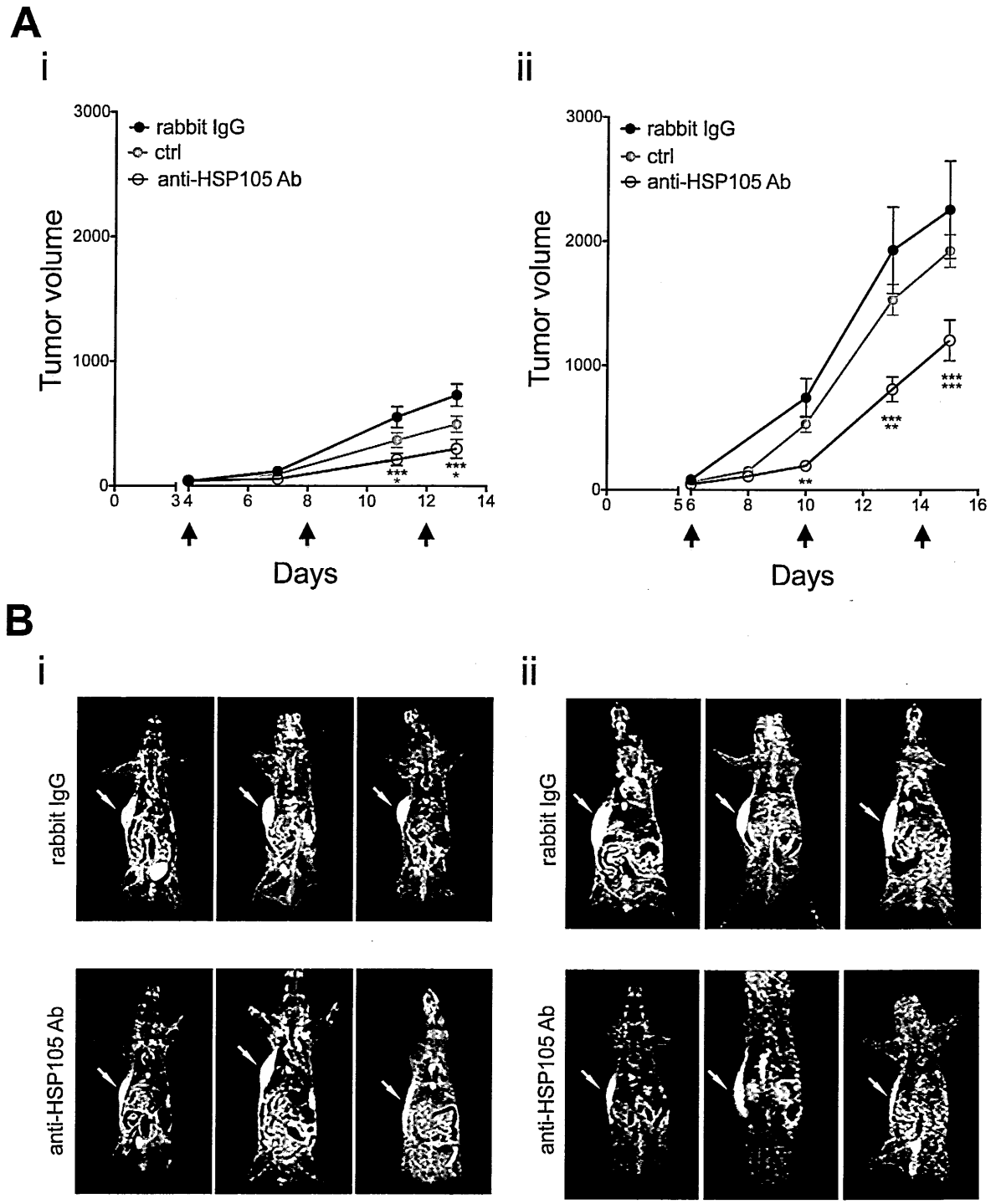


Figure 4.13 In-vivo anti-lymphoma activity of anti-HSP105 Ab.
(A) SU-DHL-4 (i) and NAMALWA growth (ii) in SCID mice treated with 3 i.p. injections of NaCl solution (ctrl), 250 μg anti-HSP105 Ab or matched isotype Igs (rabbit IgG) every 4 days (black arrows). Average tumour volumes (measured with a caliper), and SEM of 2 (SU-DHL-4 model) and 3 (NAMALWA model) independent experiments, in which 6 mice per group were analyzed, are shown. Statistically significant differences were calculated by using the 2-way analysis of variance (ANOVA) method (**: $p<0.01$; ***: $p<0.001$; black stars: anti-HSP105 Ab versus rabbit IgG; grey stars: anti-HSP105 Ab versus ctrl). (B) NMR coronal STIR images of SU-DHL-4 (i) and NAMALWA (ii) xenograft-bearing mice 24 h after the second injection of rabbit IgG (top) or anti-HSP105 Ab (bottom). White arrows indicate the hyperintense subcutaneous lesion

The greater NAMALWA tumour shrinkage compared to that of SU-DHL-4 (Figure 4.13A) was most likely due to the higher cell-surface expression level of HSP105 in

NAMALWA cells (Figure 4.8A). Importantly, the autopsy examinations confirmed the lack of detectable toxicity in the animals.

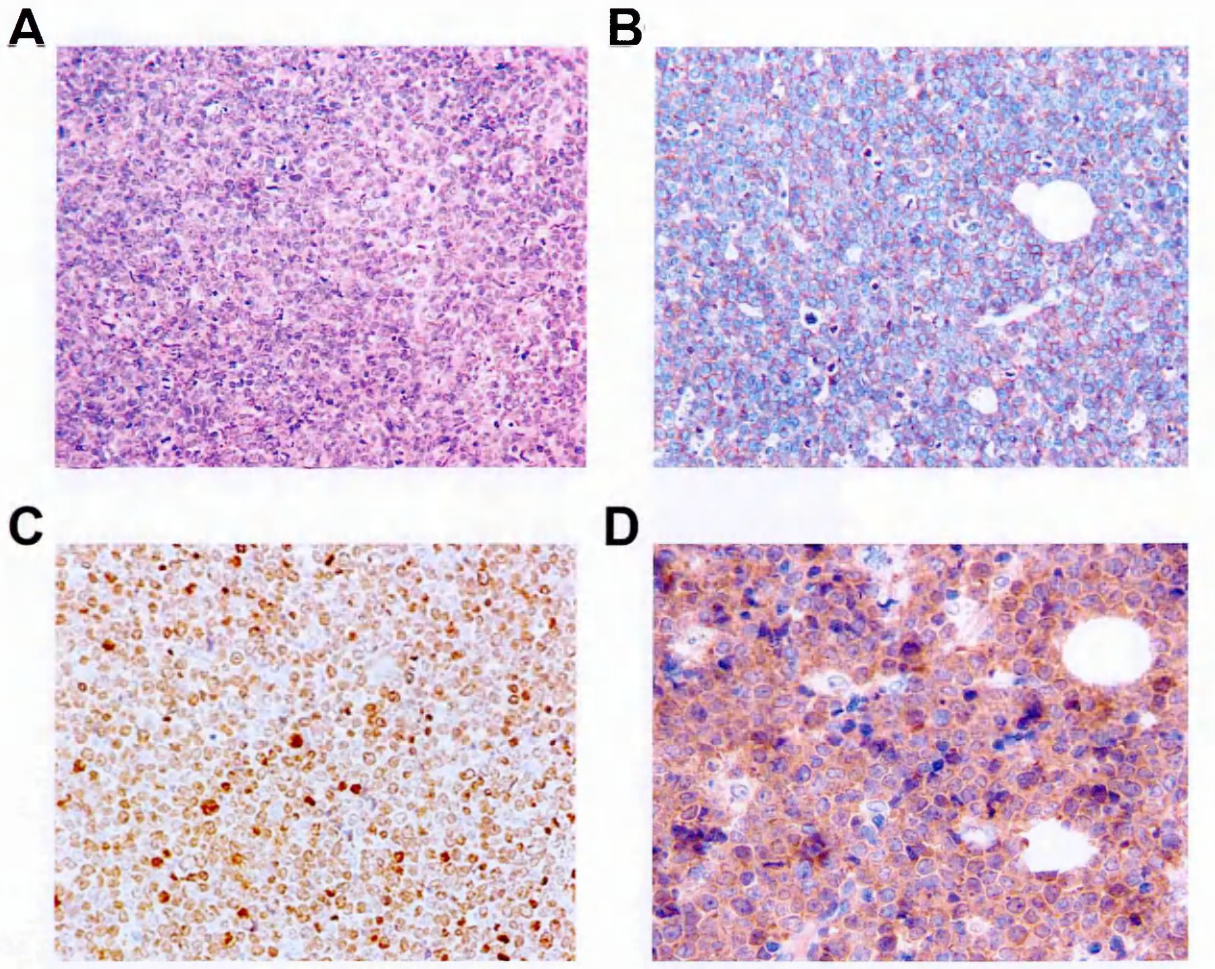


Figure 4.14 Immunihistochemical analyses of NAMALWA xenografts.
IHC analysis of NAMALWA xenografts. Hematoxylin and eosin staining (A), and IHC analyses of CD45 (B), Ki-67 (C) and HSP105 (D) expression on NAMALWA tissue sections from tumour biopsies harvested after 2 weeks of tumour engraftment in control mice. Original magnification x200 for all panels.

NAMALWA xenografts (Figure 4.14A), as defined by CD45 staining (Figure 4.14B), expressed Ki-67 (Figure 4.14C) and HSP105 (Figure 4.14D) at levels superimposable with respect to those observed in human high-grade primary B-NHL specimens (Figure 4.11Bii bottom). A marked increase of necrotic areas was detected in anti-HSP105 Ab-treated xenografts with respect to the controls, as revealed by hematoxylin and eosin stainings (Figure 4.15Ai bottom vs. top, for representative examples).

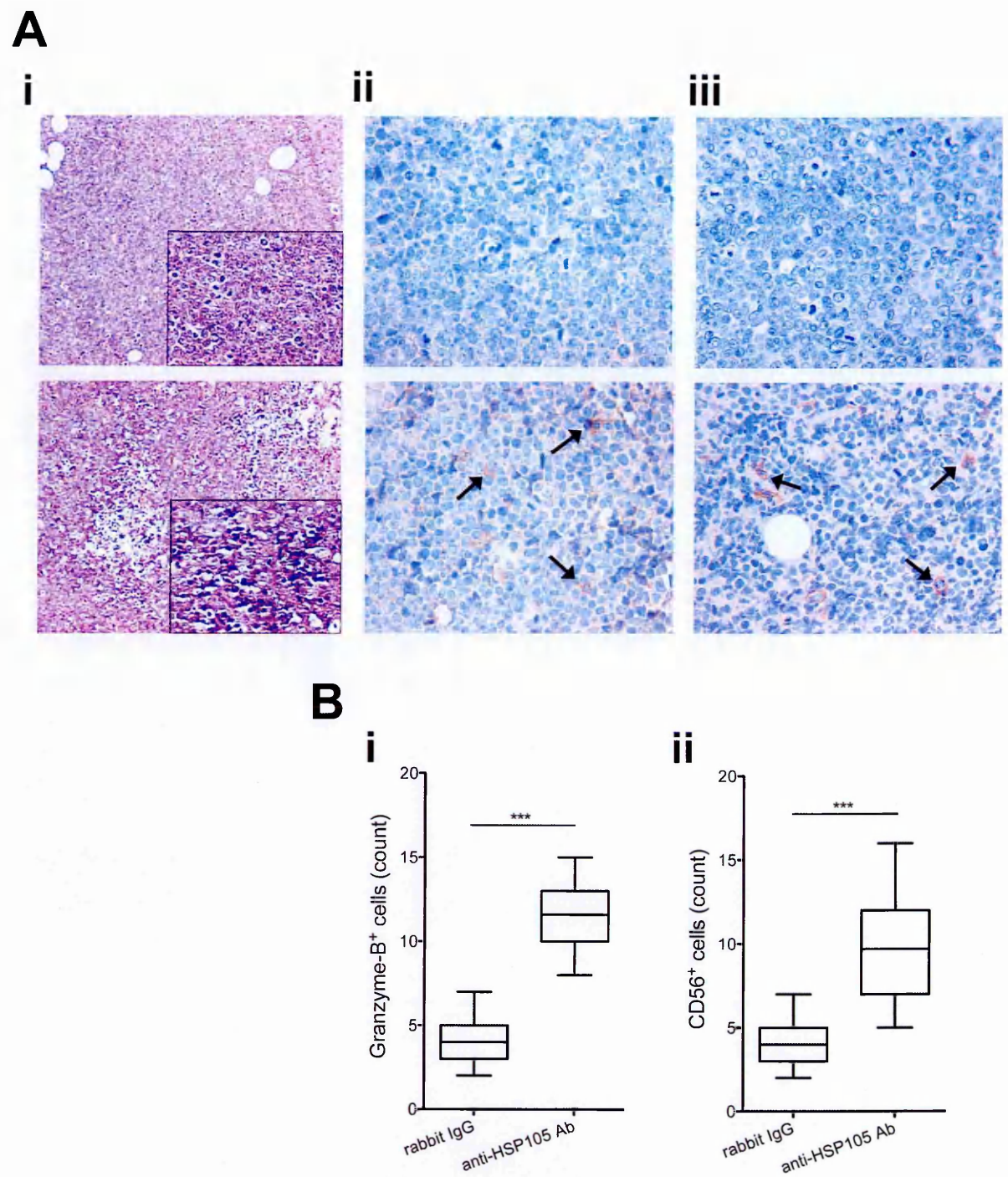


Figure 4.15 Anti-tumour effects of HSP105 targeting in the NAMALWA model.
(A) IHC analysis of NAMALWA xenografts from control rabbit Ig- (top) and anti-HSP105 Ab-treated (bottom) animals. Representative hematoxylin and eosin (i), granzyme-B (ii) and CD56 (iii) stainings are shown. Original magnification x200 (i) and x400 (ii-iii); original magnification x400 for insets. (B) Absolute number of granzyme B+ (i) and CD56+ (ii) cells in sections from control rabbit Ig- and anti-HSP105 Ab-treated xenografts. Immunoreactive cells (i.e. yellow/brown-stained cells) were counted in out of five high-power microscopic fields (x400 magnification) for each condition and the result was expressed as an average. The boxes extend from the 25th to the 75th percentiles, the lines indicate the median values, and the whiskers indicate the range of values. Results from 2 independent experiments are shown. Statistically significant differences between groups were assessed using unpaired 2-tailed Student "t" test (***: $p < 0.001$).

Under low power, untreated tumours displayed the typical "starry sky" pattern of BL, indicating the presence of apoptotic tumour cells (Figure 4.15Ai top). Upon HSP105 targeting, the tumour xenografts showed signs of single-cell necrosis and extended necrotic foci, which involved both lymphoid neoplastic cells and the branching fibrovascular

stromal network (Figure 4.15Ai bottom). Necrotic changes characterizing anti-HSP105 Ab-treated lymphomas were associated with a significant infiltration of immunological NK cells, highlighted by granzyme-B (Figure 4.15Aii bottom vs. top) and CD56 immunostaining (Figure 4.15Aiii bottom vs. top), suggesting the contribution of ADCC to the anti-tumour activity of this Ab-based immunotherapy, as already described for other Abs in different tumour models (Nagayama S, Fukukawa C et al. 2005). The quantitative evaluation of tumour-infiltrating granzyme-B+ (Figure 4.15Bi) and CD56+ cells (Figure 4.15Bii), which were counted in out of five high-power microscopioc fields in sections from anti-HSP105 Ab- and isotype Ig-treated xenografts, reproducibly confirmed this finding.

The possibility that the treatment with anti-HSP105 Ab could mediate anti-angiogenic effects was investigated in NAMALWA xenografted mice. Tumour vasculature was subjected to *in-vivo* biotin-conjugation and then quantified by IHC using HRP-conjugated streptavidin on tissue sections of the harvested tumour nodules. A significant reduction of tumour endothelial area was found in the anti-HSP105 treated mice compared to that detected in mice administered with the matched isotype Igs (Figure 4.16A, $p<0.0001$; Figure 4.16B for a representative IHC analysis). Such results may partly explain the observed anti-lymphoma activity of anti-HSP105 Ab.

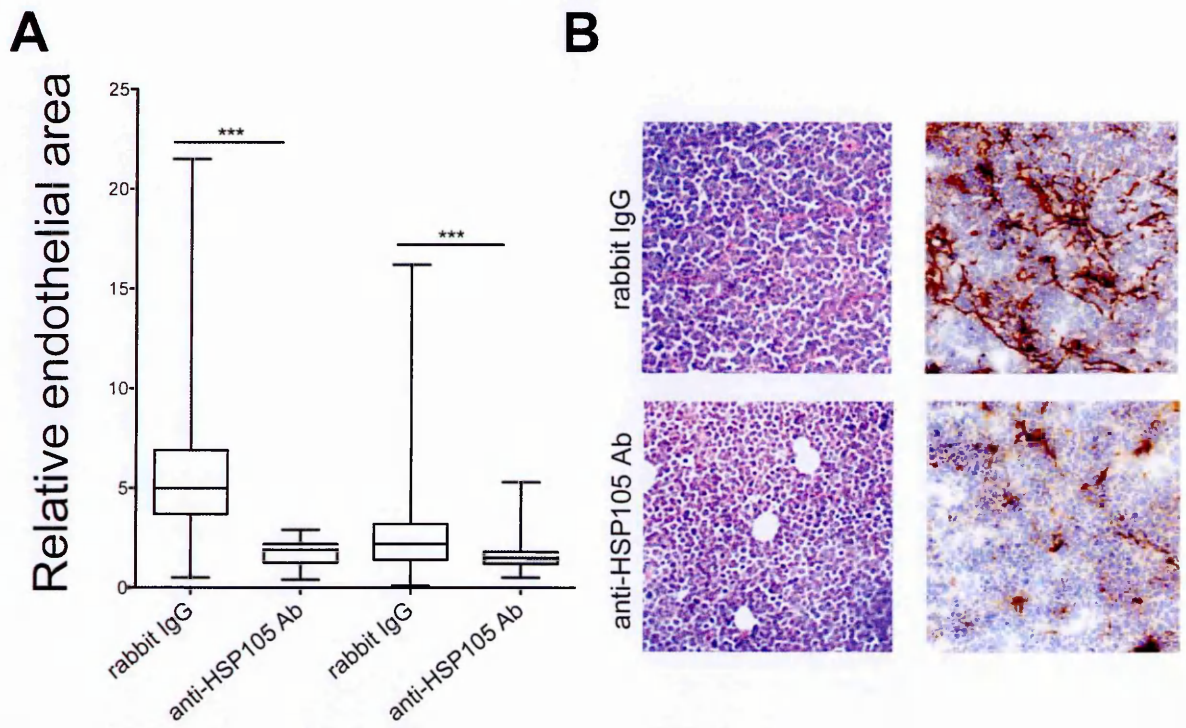


Figure 4.16 Anti-vascular effects of anti-HSP105 Ab at the tumour site.
Twenty-four hours after the second treatment, SCID mice were intravenously injected with sulfo-NHS-LC-biotin to biotinylate the tumour vasculature *in vivo*. (A) The relative endothelial area was calculated on whole tissue sections as (HRL-conjugated streptavidin stained area/total tissue area) x100. Sections obtained at 10, 20 and 30 μm in depth were analysed per tumour nodule. Nodules from 4 mice per groups were studied in two independent experiments. The boxes extend from the 25th to the 75th percentiles, the lines indicate the median values, and the whiskers indicate the range of values (***: $p<$ 0.001). (B) Representative histological images of *in-vivo* biotinylated tumour vessels in mice receiving control treatment (top) or anti-HSP105 Ab (bottom). Hematoxylin and eosin (left) and HRP-conjugated streptavidin (right) staining results are shown.

4.5 Discussion

The present study allowed the identification of HSP105 as a novel potential therapeutic biotarget for B-NHLs. Specifically, starting from the observation that DC-based vaccination in NHL patients enhanced the frequency of circulating Abs directed against shared lymphoma antigens only in Rs, an appropriately modified SERPA applied to NHL cell line proteome revealed HSP105 as one of the possible Rs' Ab-targeted proteins. The cell-surface expression of HSP105 correlated with B-NHL proliferation rate and aggressiveness, both when cell lines and primary tumour biopsies were analyzed. HSP105 targeting through a specific Ab significantly delayed and impaired the growth of aggressive human B-NHL xenografts. *In-vivo* anti-lymphoma activity of HSP105 engagement was associated with a significant increase of CD56+ and granzyme-B+ NK cells at tumour site that very likely contributed to tumour-restricted necrosis, via ADCC, and vascular damage.
Results from this study indicate the clinical relevance of anti-tumour humoral responses induced by active immunotherapy against indolent NHLs. Accordingly, previous Id-vaccination studies in FL patients reported a strong association between clinical outcome and Ab response (Timmerman 2002; Ai, Tibshirani et al. 2009). These observations are also supported by the considerable improvement in the management of B-NHLs by the addition of mAb therapy to conventional and high-dose chemotherapy protocols (Cheson and Leonard 2008). However, although the anti-CD20 mAb rituximab has significantly ameliorated the prognosis of indolent and aggressive B-NHLs (Cheson and Leonard 2008), the occurrence of resistance in about half of the patients still represents a major challenge towards the cure of these diseases (McLaughlin, Grillo-Lopez 1998; Davis, Grillo-Lopez 2000). Therefore, different and more specific biotargets as well as more potent mAbs are continuously being sought.

Vaccine-induced therapeutic Abs in patients showing a clinical response may represent a valid tool for the discovery of key TAAs, which can be exploited as biotargets for a more specific and efficient therapy. Indeed, if Abs against TAAs are commonly found in the serum of cancer patients, then, the frequency of those therapeutically targeting relevant tumour antigens is expected to increase following anti-tumour vaccination. Therefore, the serological identification of novel biotargets and/or biomarkers is likely to be facilitated by the possibility to compare the reactivity of pre- versus post-vaccine serum samples of Rs. Classical SERPA, in which cellular proteins are resolved by 2-D electrophoresis, transferred into membranes, and immunodetected using cancer patients' serum samples (Klade 2002; Hamrita, Chahed et al. 2008), has the intrinsic drawback in that it may barely detect low molecular weight proteins and those with extreme p.I., as well as low-abundance and poorly soluble hydrophobic proteins, such as cell-surface proteins. To circumvent these limitations, a 2-step approach for protein separation, including the pH gradient fractionation of protein lysates followed by 1-D SDS-PAGE of each protein fraction, has been coupled with MS analysis in the current study. Using this modified SERPA, HSP105 was identified amongst the proteins differentially revealed by Rs' post-vaccine Abs. The ability of HSP105 to elicit a humoral response has been already observed in pancreatic and colorectal carcinoma patients, whose serum samples served for its identification by SEREX (Nakatsura, Senju et al. 2001).

HSPs are ubiquitous, highly conserved proteins that function as molecular chaperones (Takayama, Reed et al. 2003). Their expression can be constitutive or regulated by multiple cellular stresses to prevent apoptosis and enhance survival. HSP105α and its alternative splicing form HSP105β belong to the HSP105/110 family, a divergent subgroup of the HSP70 family. Under physiological conditions, HSP105α is constitutively expressed and induced by various forms of stress preferentially in the cytoplasm, whilst HSP105β is expressed specifically during mild heat shock in the nucleus (Saito, Yamagishi et al. 2007; Saito, Yamagishi et al. 2009). HSP105β has been found to up-regulate HSP70 expression

trough signal transducer and activator of transcription-3 (STAT3) (Yamagishi, Fujii et al. 2009), and, like HSP105α, cooperates with HSP70 to suppress the aggregation of denatured proteins (Yamagishi 2003). Results from the present study indicate an intriguing role of HSP105 in B-cell lymphoma physiopathology, which encourage further investigation in prospective settings to evaluate whether it can truly represent a novel biomarker and/or biotarget for some categories of NHL. Interestingly, B-NHLs showed a previously undescribed surface expression of HSP105, but not HSP70 (data not shown), that increased with tumour aggressiveness and proliferation rate. As described in Chapter 3, extracellular or membrane-bound HSPs can mediate immunological functions due to their carrier function for immunogenic peptides (Suto and Srivastava 1995). Vaccines based on tumour-derived HSP70- and Gp96-peptide complexes have demonstrated potential clinical efficacy and minimal toxicity for the treatment of solid tumours as well as lymphoma and leukaemia (Srivastava 2000; Belli, Testori et al. 2002; Mazzaferro, Coppa 2003) (Janetzki, Palla et al. 2000). It is therefore possible that DC-vaccination may have provided HSP105-peptide complex to the host immune system and/or have activated it towards multiple TAAs, including HSP105, favouring the onset of a clinical response. The finding of a low cell-surface expression of HSP105 in indolent B-NHLs, however, indicates that it may not be the only relevant antigen targeted by therapeutic humoral responses induced in Rs by vaccination.

Remarkably, the administration of an anti-HSP105 Ab showed significant inhibition of the growth of human aggressive lymphoma cell lines both *in vitro* and in *in-vivo* models. The anti-tumour activity of the *in-vivo* treatment with anti-HSP105 Ab was found to be associated with significant anti-angiogenic effects. The anti-angiogenic properties of targeting the heat shock response have been already documented upon HSP90 inhibition, which leads to HIF-dependent and independent VEGF suppression and to the negative regulation of key modulators of VEGF pathway (Bohonowych, Gopal et al. 2010). Although the mechanism of the action of HSP105 targeting may be more complex, potentially affecting multiple signalling pathways downstream its client proteins and/or *in-vivo* immune effector functions (and which await further investigations) the present findings represent the proof of principle for the design and the study of anti-HSP105 passive immunotherapy for B-NHL patients.

Accumulating reports of a deep and multifaceted involvement of HSPs in the oncogenic process indeed support that the heat shock response may represent a suitable target for anticancer therapy. Due to the extreme aneuploidy, copy-number variation, and transcriptional alterations that increase the amount of toxic, unfolded protein aggregates, tumour cells need the expression of chaperone proteins to maintain normal homeostasis of the proteome (Whitesell and Lindquist 2005). Furthermore, HSPs are required for the correct maturation and function of several oncogenes, which are often chaperone client proteins. The differential expression of HSPs in tumours compared to normal cells is most likely consistent with the possibility of a sufficiently large therapeutic window for their targeting and the development of potentially efficacious rationale combination treatments (Luo, Solimini et al. 2009). A leading example in this field is provided by the experience achieved during 20 years of the study of HSP90 in cancer and 10 years of HSP90 inhibitors in clinical trials (Trepel, Mollapour et al. 2010). Its function as chaperone of oncogenes, including HER2/neu, mutant EGFR, c-KIT, BCR-ABL, BRAF, HIF1-α and, very recently, Bcl6 in DLBCL (Bohonowych, Gopal et al. 2010; Trepel, Mollapour et al. 2010) (Cerchietti, Lopes et al. 2009), and the ensuing recognition of its role as a crucial facilitator of oncogene addiction and cancer cell survival (Whitesell and Lindquist 2005) have spurred the development of specific inhibitors as novel anticancer treatment (Workman, Burrows et al. 2007). Early and more advanced clinical trials with about a number of different HSP inhibitors, representing multiple drug classes, are showing very promising results, in particular when they are combined with other targeted treatments, such as trastuzumab

(Modi et al. 2008), EGFR inhibitors (Sequist et al. 2009), bortezomib (Richardson et al. 2009), or chemotherapeutic agents (Hubbard, Erlichman et al. 2010), and/or radiotherapy (Hwang, Moretti et al. 2009).

Despite being studied to a lesser extent, the oncogenic potential of HSP105 has been already reported in solid, but not haematological, malignancies. HSP105 was indeed found to be overexpressed in thyroid, breast, gastric, esophageal, hepatocellular carcinomas and melanoma compared to normal tissues, and was thus proposed as a pertinent cancer antigen for diagnosis and immunotherapy (Nakatsura, Senju et al. 2001) (Park, Park et al. 2009). In preclinical studies, specific DNA/protein vaccination was found to be highly effective in preventing the growth of colorectal carcinoma and melanoma growth in challenged immunocompetent mice, without any signs of autoimmune reaction (Wang, Chen et al. 2003; Miyazaki, Nakatsura et al. 2005). In addition to its property as a potential target for immunotherapy, HSP105 seems to have a direct involvement in tumorigenesis, through the protection of cancer cells against apoptosis (Yamagishi, Ishihara et al. 2006; Yamagishi, Saito et al. 2008). Indeed, HSP105 siRNA treatment was found able to induce caspase-dependent apoptosis in pancreatic, colorectal and gastric cancer cell lines both *in vitro* and *in vivo* (Hosaka, Nakatsura et al. 2006).

The increasing cell-surface expression of HSP105 according to B-NHL aggressiveness and the contribution of specific Abs to the clinical efficacy of DC-vaccination, even in the setting of indolent lymphoma, strongly highlight the potential therapeutic efficacy of anti-HSP105 mAbs. The preferential expression of HSP105 in normal GC lymphocytes is consistent with recent findings showing the localization of HSP90 in normal centroblasts, thus suggesting that a stress response may govern the physiological formation of GCs (Cerchietti, Lopes et al. 2009). Although HSP105 expression was detected on the surface of normal B cells, no significant toxicity was documented after targeting HSP105 in xenografted lymphoma mouse models. This is in line with results obtained using HSP90 inhibitors that showed a good safety profile even in more extensive toxicity analyses using immunocompetent mice (Cerchietti, Lopes et al. 2009). On the other hand, the significant *in-vivo* anti-lymphoma activity of anti-HSP105 Ab treatment indicates that HSP105, or its client proteins, may represent crucial elements for tumour but not normal cell survival and supports the potential therapeutic efficacy of its selective inhibition by a specific mAb. Lymphoma have demonstrated a high sensitivity to mAb therapy, an approach that has the great advantages of a reduced off-target toxicity compared to standard chemotherapeutic regimens and also to stimulate important anti-tumour immune functions that can contribute to the long-lasting control of the disease (Hilchey, Hyrien et al. 2009). Therefore, the production of anti-HSP105 blocking mAbs may represent a good strategy to further study the therapeutic potential of targeting this molecule in B-NHL alone and in combination with conventional chemo/radiotherapy or targeted drugs to fine tune their cytotoxic effects on tumour cells and enhance their therapeutic anti-lymphoma activity.

5 OPTIMIZATION OF ANTICANCER IMMUNOTHERAPY: BOOSTING THE AFFERENT AND/OR THE EFFERENT PHASES OF VACCINE-INDUCED ANTI-TUMOUR IMMUNE RESPONSES

5.1 Introduction

Despite the promise of harnessing the immune system to target cancer cells, most phase-III trials of cancer vaccines have shown very limited clinical impact (Koos, Josephs et al. 2010) (Bendandi 2009). Importantly, the most significant clinical results have been observed only in patients treated whilst in complete response after conventional chemotherapy (Palucka, Ueno, et al. 2007; Inogès, Rodrìguez-Calvillo et al. 2006). Indeed, although active immunotherapy has repeatedly proven to boost a tumour-specific immunity into cancer patients (Neelapu, Kwak et al. 2005), it does not seem able to generate sufficient amounts of effector cells with the required functional status, homing properties and survival characteristics to overcome existing tumour escape mechanisms, particularly in the presence of a significant tumour load, and provide a therapeutically efficacious anti-tumour immune response. The relatively high clinical response rate observed in the pilot study of DC-based vaccination described in Chapter 2 is in line with the clinical efficacy achieved by DC vaccine formulations (Timmerman, Czerwinski 2002), which have the advantage to enhance multiple immunostimulatory signals, whilst promoting the down-modulation of immunoregulatory mechanisms more efficiently compared to non-DC vaccine ones (Park and Neelapu 2008; Gilboa 2007). Nevertheless, even in this case, the clinical responses were primarily achieved in patients with low tumour burden, suggesting that there may be the opportunity for further improvement of vaccine strategies to allow for the complete disease eradication and control independently of the tumour size. Recent findings in basic immunology have highlighted several new ways to potentiate both the afferent (priming) and the efferent or (effector) phases of the immune response. For example, novel immune-adjuvant agents, such as TLR ligands, or the adoptive transfer of highly avid tumour specific T cells may enhance the afferent phase of a vaccine-induced anti-tumour immune response (Wrzesinski, Paulos et al. 2007). By contrast, the efferent phase may be favoured by inhibiting various immunosuppressive and tolerance mechanisms in the tumour microenvironment, such as inhibitory co-stimulation (Attia, Phan et al. 2005) and/or Treg activity (Dannull, Su et al. 2005), as well as by stimulating pathways able to prolong the immune response elicited after a productive afferent phase (Kober, Leitner et al. 2008). These agents may be used in combination with therapeutic cancer vaccines for optimal induction of anti-tumour immunity that, in turn, may lead to improved clinical outcome.

Manipulation of the immune system by adoptive transfer of tumour-specific lymphocytes, activated and expanded *in vitro*, has shown remarkable clinical results in cancer patients (Rosenberg, Yannelli et al. 1994; Haque, Wilkie et al. 2007; Bollard, Aguilar et al. 2004) (Dudley and Rosenberg 2003), and in combination with chemotherapy has been demonstrated to mediate significant tumour regression in heavily pre-treated metastatic melanoma patients with conspicuous tumour burden (Dudley, Wunderlich, et al. 2005). However, this strategy is technically demanding; for example, it requires a lot of time for the generation of large amounts of anti-tumour T cells for every patient (Gattinoni, Klebanoff et al. 2005). Furthermore, on reaching the terminal stages of differentiation after long-term *ex-vivo* stimulation, most expanded T cells exhibit a limited capacity to further divide and persist *in vivo* (Yee, Thompson et al. 2002; Gattinoni, Klebanoff et al. 2005; Hinrichs, Borman et al. 2009; Robbins Dudley et al. 2004). Improvements to efficiently boost the rapid expansion of functional anti-tumour T cells possessing extensive replicative capacity and long-term *in-vivo* persistence are required to better exploit the therapeutic

potential of adoptive immunotherapy as a tool to enhance the afferent phase of an anti-tumour immune response into cancer patients.

The increased appreciation of the relevance of T cell co-stimulatory pathways in fine-tuning an efficient immune response and the concurrent development of the related agonist or antagonist agents have provided novel strategies to favour the establishment of anti-tumour immunity (Croft 2009). In particular, OX40 (CD134) and its partner OX40L (CD252), both members of the tumour necrosis factor receptor (TNFR)/TNF superfamily, provide one of the most important and prominent interactions (Croft 2010; Ishii, Takahashi et al. 2010). OX40-OX40L co-stimulation has the ability to sustain an ongoing immune response by inducing cell division and survival signals to differentiating or already differentiated T cells, and by enhancing the production of differentiating cytokines to further polarize the immune responses (Croft 2010; Ishii, Takahashi et al. 2010). Thus, this interaction not only regulates the magnitude and the efficiency of the primary response, but also the generation of the T-cell memory pool. In addition, OX40 stimulation has been recently found to inhibit existing Tregs and block their peripheral conversion in basic and tumour mouse models (Valzasina, Guiducci et al. 2005; Kroemer, Xiao et al. 2007; Piconese, Valzasina et al. 2008). These properties, together with the restricted expression of OX40 on antigen activated T cells, make it an attractive and promising molecular target to be stimulated in combinatorial immunotherapeutic approaches, including DC vaccines, ACT, and treatment with cytokines (GM-CSF and IL-12).

5.2 Aim of the Chapter

The major aims of this Chapter were the following:

- To develop a new expansion system for the production of large amounts of anti-tumour T cells with an extensive replicative capacity and long-term *in-vivo* persistence potential. Towards this goal, the liposome-based artificial APCs (aAPCs) described by Albani and colleagues (Prakken, Wauben et al. 2000; Giannoni, Barnett et al. 2005) for the identification of rare antigen-specific T cells in autoimmune disorders were properly modified for *ex-vivo* T-cell activation and expansion. Artificial liposome-based systems are expected to preserve the properties of a fluid cellular membrane that allows for efficient immunological synapse formation, whilst providing highly reproducible stimulation that can be tightly regulated by the addition of the desired type and amount of immunomodulatory molecules;

- To study OX40-OX40L interactions in the lymphoma microenvironment and verify the possibility of exploiting this pathway as a potential target for immunotherapy for NHLs. The expression of OX40 was analysed in lymphoma cell lines and primary tumours, as well as in T cells and Tregs in PB and at the tumour site in NHL patients. The effects of blocking or stimulating OX40 on human T cells and Treg were also investigated *in vitro*.

5.3 Materials and Methods

5.3.1 Generation of aAPC

For the preparation of aAPCs, the approach used by Albani and colleagues (Prakken, Wauben et al. 2000; Giannoni, Barnett et al. 2005) was adopted with some modifications. Briefly, mouse anti-human CD3 (clone UCHT1, isotype IgG1k; BD Pharmingen), CD28 (cloneCD28.2, isotype IgG1k; BD Pharmingen) and LFA1 (clone 38, isotype IgG2a; Biodesign, Saco, MN) triggering mAbs were pre-clustered in microdomains (MDs) on a scaffold made of GM1 ganglioside-enriched liposomes (Figure 5.1A). To this end, biotinylated anti-CD3, anti-CD28 and anti-LFA1 mAbs were combined with biotinylated cholera toxin B (CTB; Sigma) in a 3:1 molar ratio. After 5 min incubation, neutravidin (NA; Pierce Biotechnology, Rockford, IL) was added at a ratio of 1 mol per 4 biotinylated moieties for an additional 15 min. These reagents were then mixed with GM1-liposomes (kindly provided by Dompè Biotec Spa, L'Aquila, Italy) after the removal of detergent by dialysis for 72 hours against PBS in a 10K Slide A Lyzer (Pierce Biotechnology, Rockford IL). After mixing for 90 min incubation, the aAPC solution was spun in PBS at 14,000 rpm for 10 min. The pellet containing the aAPC was resuspended in culture medium and used for T cell expansion. As controls, T cells were cultured in parallel in the presence of the liposome formulation (liposome alone) or biotinylated mAbs bound on NA rafts (MD alone) alone. To validate the efficacy of the association of the three mAbs on aAPC, initial experiments were performed using aAPC whose MDs contained only one (anti-CD3, or anti-CD28, or anti-LFA1) or two (anti-CD3 and anti-CD28, anti-CD28 and anti-LFA1, anti-CD3 and anti-LFA1) biotinylated mAbs. CFSE- (Invitrogen) stained PBMCs from healthy donors were stimulated with aAPC containing one of the following combinations of mAbs: anti-CD3/-CD28/-LFA1 (hereafter named standard aAPC), anti-CD3/-CD28, anti-CD3/LFA1, anti-CD28/-LFA1, anti-CD3 alone, anti-CD28 alone, anti-LFA1 alone. For each combination of mAb, aAPC were produced with two-fold serial dilutions (range 1/1 to 1/64) of the standard mAb concentration. CFSE dilution was assayed after 4, 7 and 10 days using a FACSCalibur flow cytometer and results were analysed with CellQuest software (Becton Dickinson). In addition, the T-cell expansion efficiencies of complete aAPC and those lacking anti-LFA-1 mAb were tested in long-term cultures (14 and 28 days).

5.3.2 Human cells and culture conditions

Written informed consent was obtained from healthy donors and patients whose materials were used in this study after Institutional Review Board approval. Two sources of T cells were tested for the expansion: (i) CD3+ lymphocytes, negatively isolated from healthy donors' PBMCs, obtained by Ficoll-Hypaque density gradient separation of heparinised blood, which were prepared using a Pan T cell isolation kit and a MiniMACS device (Miltenyi Biotech), according to the manufacturer's protocol; and (ii) lymphocytes from HLA-A*0201 melanoma patients isolated from LN metastasis, which were cultured for 2 weeks with HLA-A*0201+ T2 cell line loaded with 10 μg/ml of Melan-A/MART-1 27-35 (modified sequence 27–35: ELAGIGILTV; PRIMM s.r.l.) as described (Anichini, Molla et al. 1999). Lymphocytes from the melanoma-associated LNs were analysed for the frequency of MART-1-tetramer+CD8+ T cells before and at the end of culture.

T cells were seeded at a concentration of 0.2 x 10^6 cells/ml in a 96 flat-bottomed well plate with 2050 μl/well of RPMI 1640 (Lonza) containing 10% human serum, 1% PenStrep (Lonza), 1% Glutamine (Lonza) and 1% Hepes buffer (Lonza). A single stimulation with aAPC, or mouse anti-human CD3/CD28 mAb-coated immunomagnetic microbeads (Figure 5.1B, Dynabeads® CD3/CD28 T Cell Expander or Dynabeads® ClinExVivo CD3/28, Dynal Biotech ASA, Oslo, Norway), or purified mouse anti-human CD3

mAb (OKT3 clone, eBioscience)-coated well plate (Figure 5.1C) was used to assess T-cell expansion. Anti-CD3 mAb was cross-linked to the well bottom of 96-flat bottomed well plates by 45 min incubation at a concentration of 0.5 μg/ml in RPMI 1640 (50 μl/well) after pre-coating the plate for an overnight with 10 μg/ml anti-mouse IgG (whole molecule, Sigma). Before seeding the T cells, the cross-linking reaction was stopped by adding 20% human serum-supplemented RPMI 1640 (50 μl/well). After stimulation with one of these systems, cultures were grown in the presence of the following combination of γ-chain cytokines according to previous results (Anichini, Mortarini et al. 2006a): (i) low dose (LD) recombinant human rhIL-2 (300UI/ml, Proleukin, Chiron, Emeryville, CA); (ii) rhIL-15 (10 ng/ml, Peprotech Inc., Rocky Hill, NJ); (iii) high dose (HD) rhIL-2 (3,000 UI/ml) plus rhIL-15 10 ng/ml. Complete culture medium with cytokines was replaced every 2 days during expansion. For some experiments, aAPC were generated with 1 or 2 log reduced concentrations of biotinylated mAbs and tested for the expansion of CD3+ purified T cells in comparison to the standard ones in association with HD IL-2 and IL-15. In addition, standard aAPC were used for the expansion of 10- (0.5 x 10^6 T cells/aAPC dose) or 100-fold (5 x 10^6 T cells/aAPC dose) increased starting cell number, in 1 or 4 ml culture medium per 48- or 12-well plate well, respectively, in the presence of HD IL-2 plus IL-15. Complete culture medium with cytokines was replaced every 2 days during expansion.

5.3.3 Flow cytometry analysis

T-cell maturation and activation phenotypes were evaluated by flow cytometry using the following mouse anti-human mAbs in different combinations: FITC-labelled anti-CD3, PE-labelled anti-CD25 (Miltenyi Biotech), FITC-labelled anti-CD62L, FITC-labelled CD27, PE-labelled anti-CD45RA, PE- or PerCP-labelled anti-CD4, anti-CD69 and anti-CD3, APC-labelled anti-CD8 and anti-CD4 (BD Biosciences), and APC labelled anti-CCR7 (R&D systems, Minneapolis, MN, USA). To detect antigen-specific T cells directed to Melan-A/MART-1, lymphocytes were stained with PE-labelled tetramers of HLA-A 0201 containing Melan-A/MART-127-35 peptide (Beckman Coulter Inc., Fullerton, CA, USA). Surface staining was performed by incubating the cells with the mAbs at 4°C for 30 min. Tregs were characterized by the expression of intracellular FOXP3 in association with the following mouse anti-human mAbs in different combinations: PE-labelled anti-CD25, PerCP-labelled anti-CD8 or anti-CD4, APC-labelled anti-CD4 or anti-OX40 (eBioscience) mAbs, as described in Chapter 2. APC-labelled anti-OX40 (eBioscience) and FITC-labelled anti–OX40L (Ansell) mAb co-staining was performed on human NHL cell lines listed in the Material and Methods section of Chapter 4 (paragraph 4.3.1), and in donors' PBMCs or normal reactive and malignant LNs in combination with the following mAbs: PE-labelled anti-CD19, -CD20 or -CD4, PerCP-labelled anti-CD45, -CD3 or –CD8 mAbs (BD Biosciences). Apoptosis was assayed by staining with FITC-labelled annexin V (ANN V) and propidium iodide (PI) (rh Annexin V/FITC Kit, Bender MedSystem, Vienna, Austria), according to the manufacturer's protocol. To detect intracellular perforin or Granzyme B in T cells after 14 days of stimulation with aAPC, microbeads or immobilized anti-CD3 mAb, expanded T cells were permeabilized with Cytofix/Cytoperm (BD Biosciences) and then stained with FITC–labelled mouse anti-human perforin (BD Biosciences) or PE–labelled mouse anti-human Granzyme B (CLB, Amsterdam, The Netherlands) in the presence of Perm/Wash solution (BD Biosciences). The binding of aAPC on T cells was detected by staining with FITC-labelled goat anti-mouse IgG mAb (Jackson ImmunoResearch) at days 7 and 14 after stimulation. To assess the sensitivity of this assay, a fixed amount (5×10^4) of human CD3+ T cells were incubated with the indicated dilution of the anti-CD3, anti-CD28, and anti-LFA-1 mAb standard concentration used to prepare as many aAPC as required for the stimulation of such an amount of cells. The MFI obtained from the staining with the secondary Ab were then plotted against the known concentrations of anti-CD3, anti-CD28 and anti-LFA-1 mAbs to obtain a titration curve. The amount of mAbs, and indirectly of

aAPC, still present on T cells at days 7 and 14 after stimulation, were thus extrapolated for the experimentally derived MFI.

All stained samples were acquired on a FACSCalibur using the CellQuest software (Becton Dickinson). Data were subsequently analysed using FlowJo software.

5.3.4 Cytotoxic assay

The cytotoxic activity of expanded MART-1 specific T cells was assessed through ^{51}Cr-release assay using as target an HLA-A*0201+ lymphoblastoid cell line (LCL 9742) loaded with Melan-A/MART-127-35 peptide. Negative controls were represented by unloaded LCL or LCL loaded with Influenza A (Flu) Matrix58-66 (GILGFVFTL) or HIV (ILKEPVHGV) produced by PRIMM s.r.l.. Synthetic peptides were ≥95% pure (Anichini, Molla et al. 1999). Results were expressed as follows:

% Lysis = (experimental release (cpm) - spontaneous release (cpm))/ (maximum release (cpm) - spontaneous release (cpm)) x 100

where spontaneous release was assessed by incubating target cells in the absence of effectors and maximum release was determined in the presence of 1% Nonidet P40 detergent (BDH Biochemical, Poole, UK).

5.3.5 Purification of the CD4+CD25+ and CD4+CD25- T-cell subsests and the CFSE-based proliferation assay

PBMCs isolated by Ficoll-Hypaque density gradient from donors' or lymphoma patients' heparinised PB or cell suspensions obtained from mechanic dissociation of malignant or reactive LN were used as source of CD4+CD25+ Tregs and CD4+CD25- effector T cells (Teffs). These subpopulations were purified using CD4+CD25+ Treg isolation kit (Miltenyi Biotec), according to manufacturer' s instruction. Flow cytometry showed that the separate fractions were ≥ 90% pure. For *in-vitro* assays, purified CD4+CD25- Teffs were labelled by incubation with 5 μM CFSE (Invitrogen) in PBS containing 5% FBS for 15 min at 37°C. Cells were washed twice with PBS and set up in a proliferation assay. To test Treg immunosuppressive activity, 10 or 5×10^4 CFSE-labelled CD4+CD25- Teffs were seeded, respectively, alone or with 5×10^4 CD4+CD25+ Tregs in each well of a 96-well flat-bottom plate in complete medium supplemented with 1μg/ml PHA (Sigma), to stimulate Teff proliferation through TCR engagement. In some experiments, an anti-OX40 neutralizing mAb (ACT35, eBioscience) or human recombinant OX40L fusion protein (FcIlzOX40L, kindly provided by Dr Weinberg) was added at the indicated doses. To verify the ability of OX40L recombinant protein to promote human T-cell survival and proliferation, its activity was tested in a soluble assay as described by Morris and colleagues (Morris, Peters et al. 2007), with minor modifications. Briefly, CFSE-labelled Teffs were initially stimulated for 48 hours in the presence of 1 μg/ml PHA and 10 UI rhIL-2 to boost their expression of OX40, then they were seeded in 96-well flat bottom plate, pre-coated with two-fold serial dilution of anti-CD3 mAb (OKT3, range 0.3-40 μg/ml), in the presence of 10 μg/ml FcIlzOX40L. After 48- and 72-hour incubation, T-cell proliferation and survival was monitored by flow cytometry analyses of, respectively, CFSE dilution and 7-aminoactinomycin D staining (7-AAD, Invitrogen) performed at a concentration of 1μg/ml in 2% fetal bovine serum PBS for 20 min-incubation at 4°C.

5.3.6 Statistical analysis

Statistical significance was determined using the two-sided Student's "*t*" test. Regression analysis was conducted on GraphPad Prism version 5 for Mac software (GraphPad Software, La Jolla, CA).

5.4 Results

5.4.1 aAPC with pre-clustered anti-CD28/-CD3/-LFA-1 mAbs are highly effective in inducing the ex-vivo expansion of functional human anti-tumour T cells

5.4.1.1 Generation of aAPC and expansion of human polyclonal T cells

As shown in Figure 5.1A, aAPC consist of a scaffold made of GM1-enriched liposomes, with anchor MDs containing the agonist biotinylated mAb (anti-CD3, anti-CD28, and anti-LFA1) through a biotinylated CTB subunit and NA.

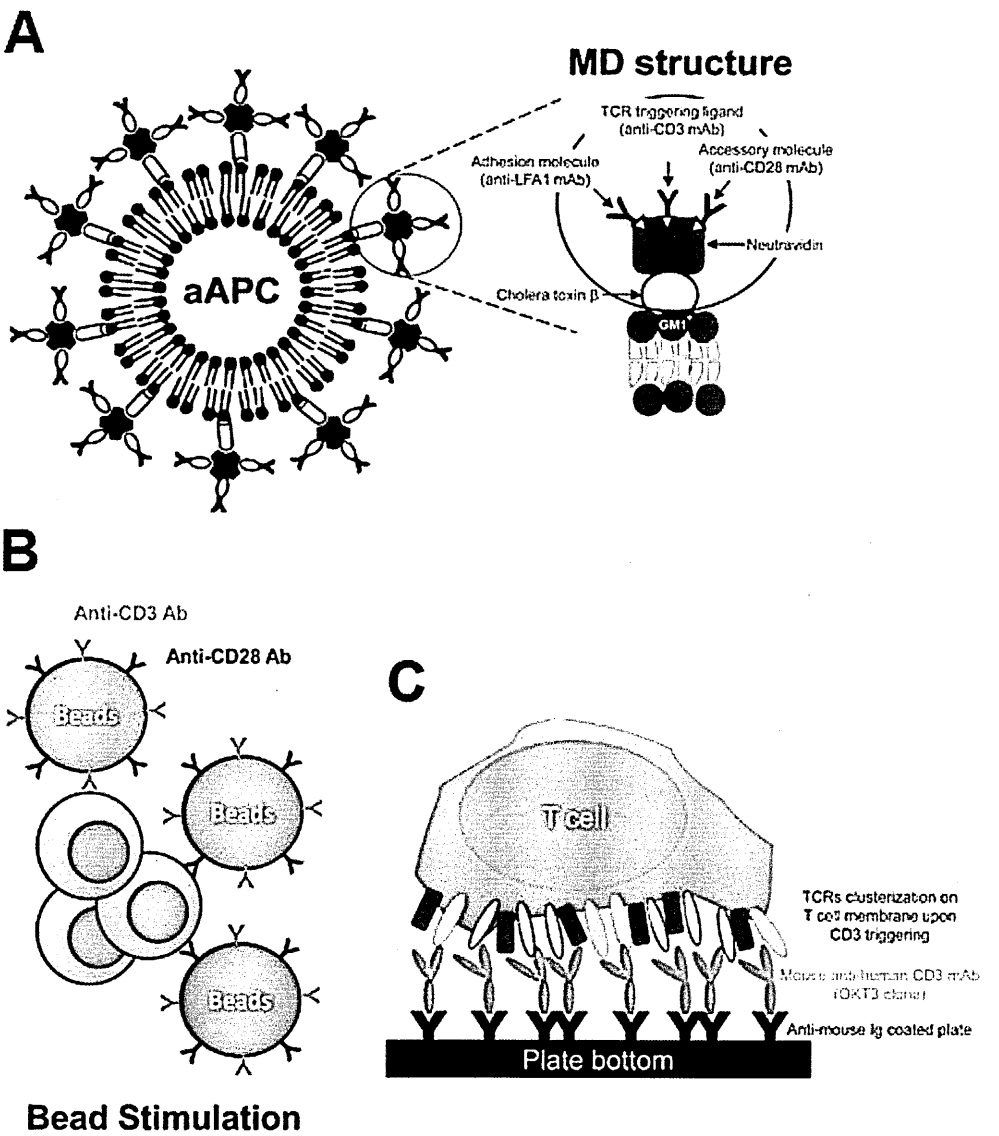


Figure 5.1 Structures of artificial systems tested for T cell expansion.
(A) Representation of the MD structure carried by aAPC (adapted from Giannoni 2005). The lipid bilayer was enriched with GM1 ganglioside to form a bridge with biotinylated cholera toxin B (CTB). In each MD, one molecule of neutravidin (NA) anchored biotinylated anti-CD3 and anti-CD28 and anti-hLFA-1 agonist mAbs to the aAPC surface through one biotinylated CTB. (B) Schematic representation of Dynabeads® CD3/CD28 T cell Expander. Anti-CD3 and -CD28 triggering mAbs on immunomagnetic beads can interact with and activate the cognate molecules on T cells. (C) Schematic representation of mouse anti-human CD3 mAb (OKT3 clone) cross-linked through anti-mouse IgG to the plate well bottom. The exposed anti-CD3 mAb Fab domains interact with TCRs and activate T cells.

Purified polyclonal CD3+ T cells (mean purity 90±3%), when stimulated with aAPC containing all the three agonist mAbs, displayed a higher proliferation rate compared to those cultured in the presence of aAPC lacking anti-LFA-1 mAb, after both 14- and 28-day incubation (Figure 5.2).

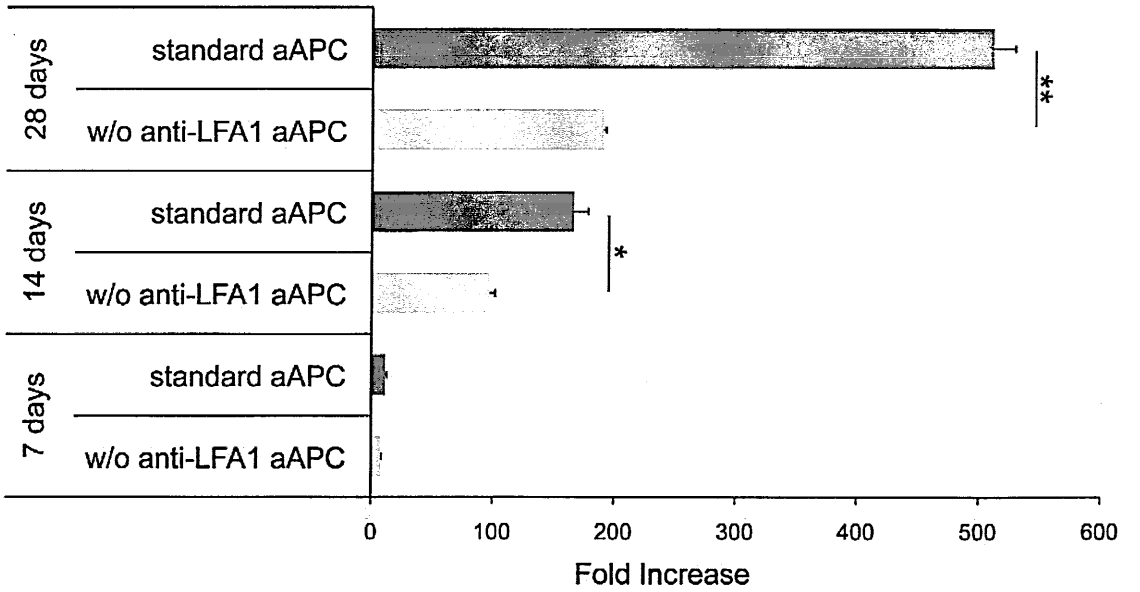


Figure 5.2 Long-term expansion experiments.
Comparison of T-cell expansion after 7, 14 and 28 days from a single stimulation with complete aAPC or anti-CD28/-CD3 aAPC (w/o anti-LFA1 aAPC) in the presence of HD IL-2 and IL-15. Fold increase values were calculated on the basis of the percentage of ANN V−/PI− viable cells detected by flow cytometry (*: $p \leq 0.05$; **: $p \leq 0.01$) and the absolute cell count obtained by the Tripan blue exclusion test..

For this reason, aAPC containing the mixture of all three mAb were used in the subsequent experiments. They were tested in association with LD IL-2, or IL-15 or HD IL-2 plus IL-15 for the *in-vitro* expansion of human polyclonal CD3+ T cells. In parallel, T cells were cultured with liposomes or with MDs alone as controls. After 7 days of stimulation, the greatest efficiency was observed when complete aAPC were associated with any cytokine combination. However, after 2 weeks, aAPC stimulation with HD IL-2 plus IL-15 resulted in the greatest fold increase in T cell number, which was significantly higher with respect to the other conditions (Figure 5.3).

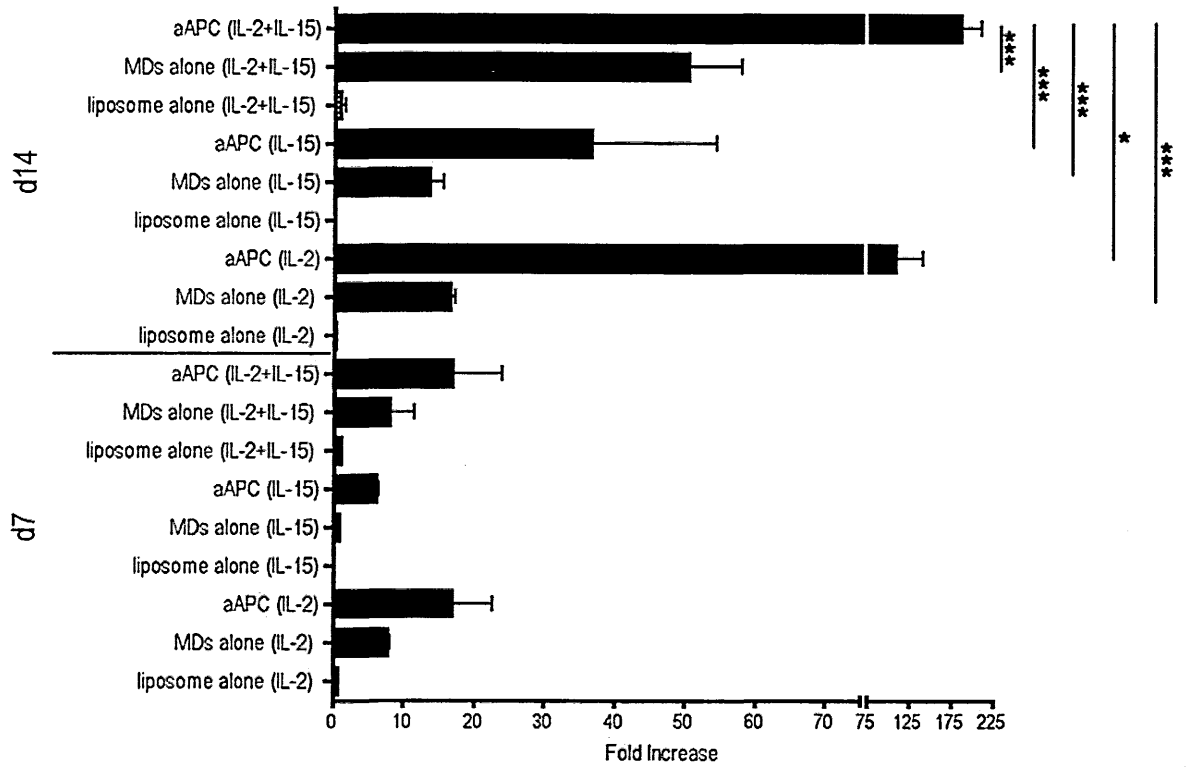


Figure 5.3 Activity of complete aAPC compared to their single components.
Fold increase in viable T-cell number measured by the Tripan blue exclusion test after 7 and 14 days of culture of freshly purified donors' polyclonal CD3+ T cells stimulated with aAPC, MDs or liposomes in the presence of LD IL-2, or IL-15, or the combination of HD IL-2 and IL-15. Results are representative of four independent experiments with different donors. Error bars indicate standard deviation of the mean. Statistically significant differences, calculated using two-tailed Student *t* test, are reported (*: $p \leq 0.05$; ***: $p \leq 0.001$).

MDs alone provided a positive stimulus for T-cell proliferation, since they consisted of the biotinylated activating mAbs grouped on NA. However, placing them into a lipid membrane allowed their activity to be efficiently oriented, increasing the final stimulus for T-cell expansion. Liposomes alone did not provide any positive stimulations for T-cell expansion (Figure 5.3).

5.4.1.2 Survival of human polyclonal T cells after stimulation with aAPC

The activity of aAPC was then compared with that provided by the other commercially available artificial systems for T-cell expansion (anti-CD3 and -CD28 mAb-coated immunomagnetic microbeads, Dynabeads® CD3/CD28 T Cell Expander and immobilized anti-CD3 mAb, clone OKT3). Freshly purified donors' polyclonal CD3+ T cells were stimulated with one of these systems and expanded in the presence of IL-2 and/or IL-15. After 2 weeks of incubation, microbeads and aAPC in combination with HD IL-2 plus IL-15 provided the greatest T cell expansion efficacy (Figure 5.4A).

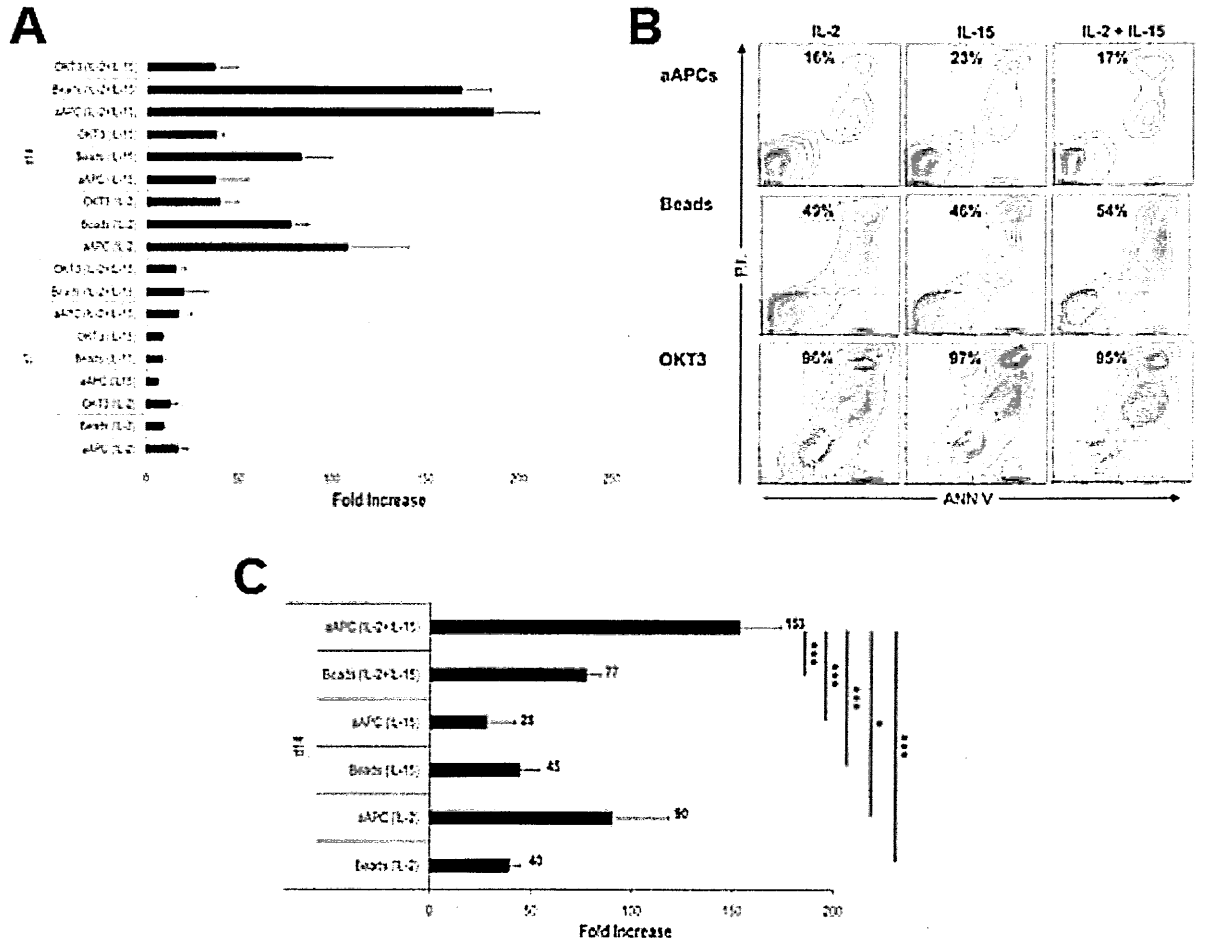


Figure 5.4 Activity of aAPC compared to commercial artificial systems.
(A) Fold increase in donors' polyclonal CD3+ T-cell number after stimulation with aAPC, immobilized anti-CD3 mAb or anti-CD3/-CD28 microbeads in the presence of LD IL-2, or IL-15, or the combination of HD IL-2 and IL-15. Viable cell counts were assessed using the Tripan blue exclusion test after 7 (d7) and 14 (d14) days of culture. Results are representative of four experiments with different donors. Error bars indicate the standard deviation of the mean. (B) Representative flow cytometry analysis of apoptosis of the same cultures shown in A after 14-day expansion. FITC-labelled ANN-V (x-axis) and PI (y-axis). In each plot the frequency of total dead cells is reported (C) Fold increase in viable ANN-V-/PI- T-cell number after 14-day expansion with aAPC or anti-CD3/-CD28 microbeads in the presence of LD IL-2, or IL-15, or the combination of HD IL-2 and IL-15. Error bars indicate the standard deviation of the mean. *p* values were calculated using the two-tailed Student's *t* test (*: $p \leq 0.05$;***: $p \leq 0.001$).

However, flow cytometry analysis of apoptosis revealed a significant increase of cell death in cultures stimulated for 14 days with microbeads or OKT3 in the presence of any cytokine combinations tested (Figure 5.4B). In the bead-stimulated culture, only 46% of T cells expanded with IL-2 and Il-15 were viable (ANN V–PI-), whereas 83% of T cells were still alive 14 days after the stimulation with aAPC. Accordingly, this system provided a significantly greater expansion of viable ANN V-PI- T cells compared to that afforded by the other artificial systems (Figure 5.4C). As a further control, anti-CD3/-CD28 microbeads were compared to aAPC lacking anti-LFA-1 mAb, to evaluate the T-cell expansion efficacy of the two systems, when the triggered molecules on T cells were the same. Even in these conditions, aAPC were able to expand a higher amount of T cells without affecting their viability (Figure 5.5).

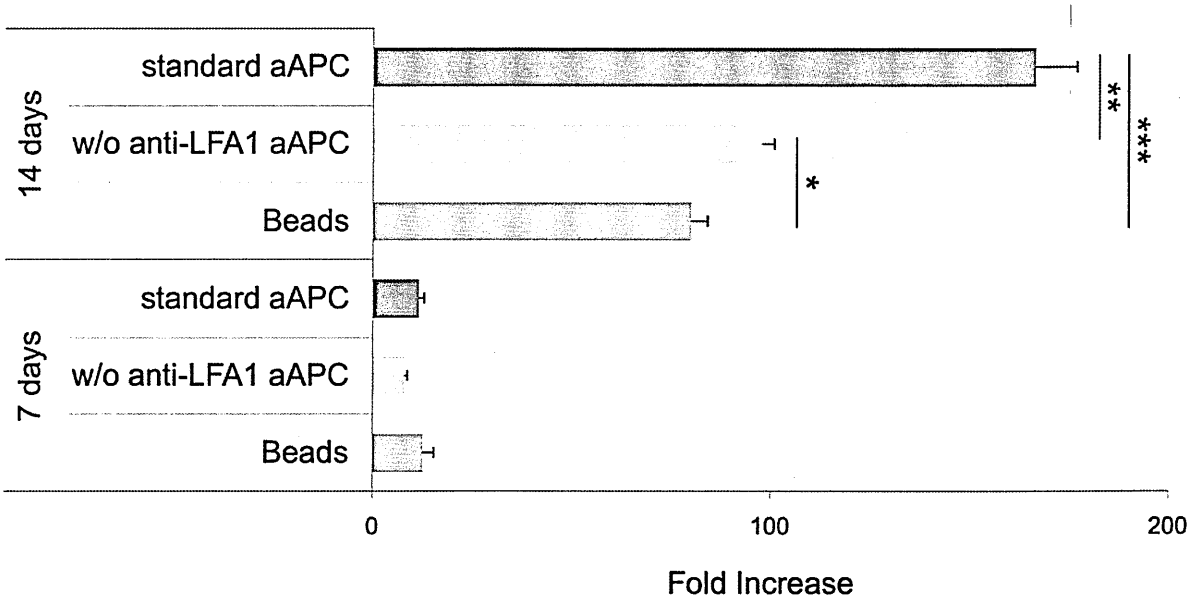
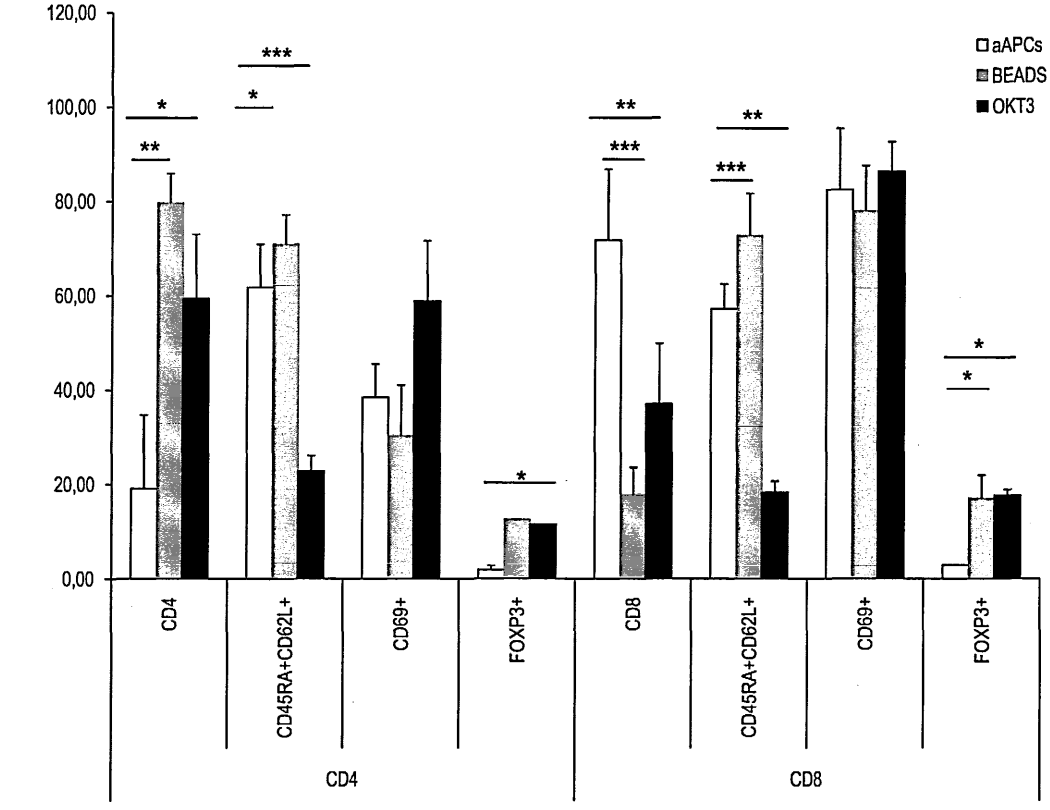


Figure 5.5 Anti-CD28/-CD3 aAPC vs. anti-CD28/-CD3 microbead T cell expansion.
Expansion efficiencies of the polyclonal CD3+ T cells of donors achieved by a single stimulation with complete aAPC, anti-CD28/-CD3 aAPC (w/o anti-LFA1 aAPC) or anti-CD28/-CD3 microbeads in the presence of HD IL-2 and IL-15 for 14 days of culture. Fold increase values were calculated on the basis of the percentage of viable ANN V−/PI− T cells, as detected by flow cytometry analysis of apoptosis, and the absolute cell count obtained with the Tripan blue exclusion test (*: $p \leq 0.05$; **:$p \leq 0.01$; *** $p \leq 0.001$).

These results demonstrated the stimulation advantages provided by an artificial system based on a fluid membrane to carry triggering molecules for the activation of the cognate ligands on T cells. In addition, including anti-LFA-1 mAb amongst the stimulating moieties on the aAPC allowed for a further increase of T-cell expansion activity (Figure 5.5).

5.4.1.3 Immunophenotype of expanded polyclonal T cells

The immunophenotypic analysis, carried on after 14-day expansion, interestingly revealed an inverse ability of aAPC compared to microbeads to expand CD8+ and CD4+ T cells (Figure 5.6A), with aAPC preferentially increasing the frequency of the former in culture. In contrast to adherent anti-CD3 mAb stimulation, these systems provided comparable high levels of naïve and effector cell enrichment in both T cell subpopulations, with beads favouring a significantly higher expansion of CD45RA and CD62L double-positive T cells (Figure 5.6A-B).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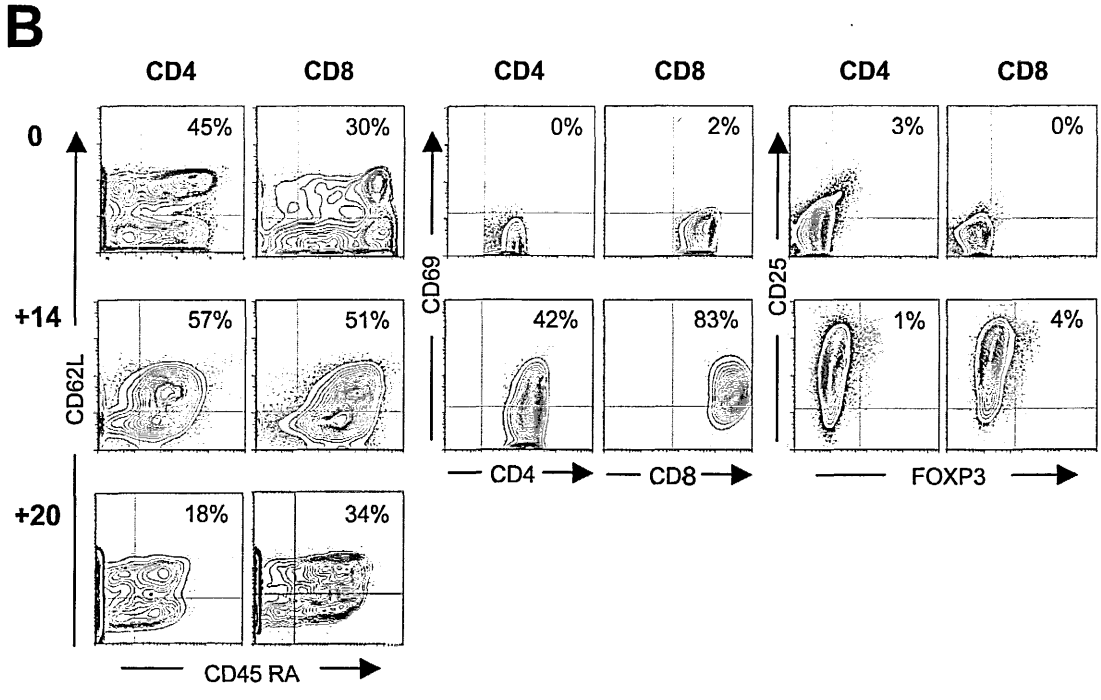
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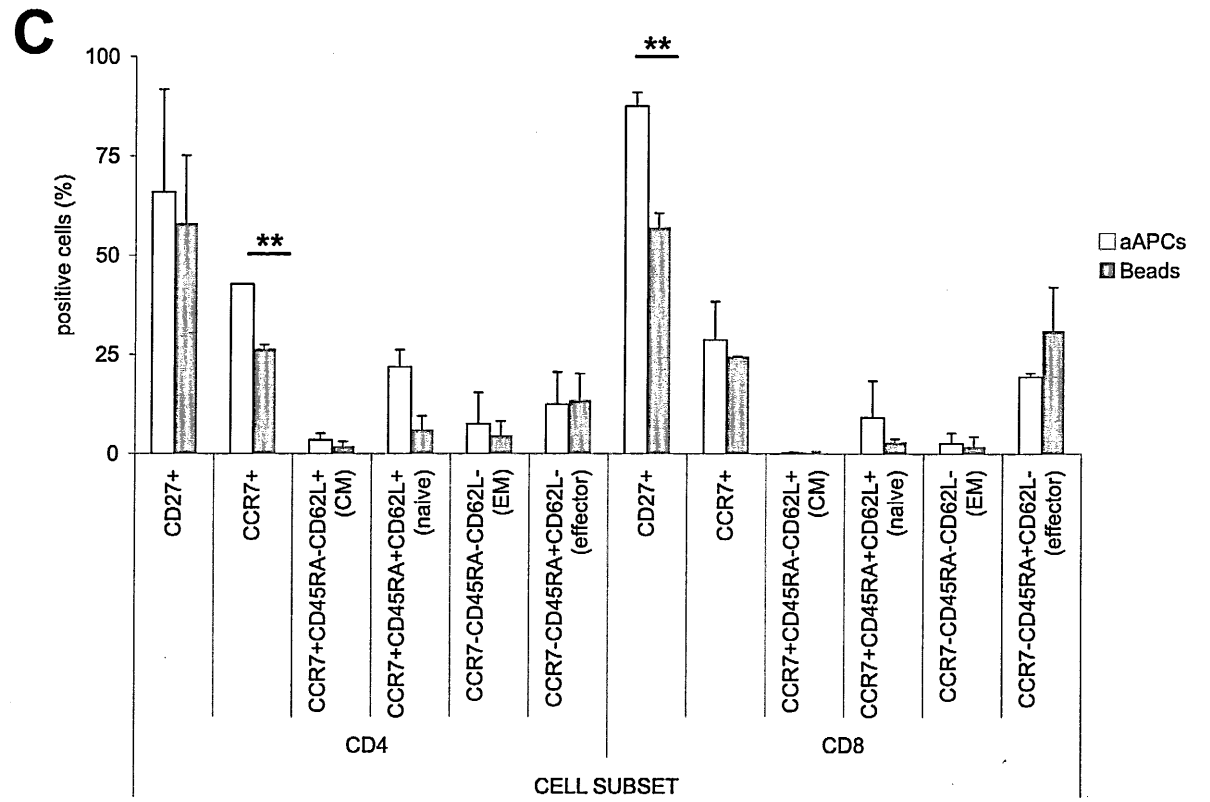


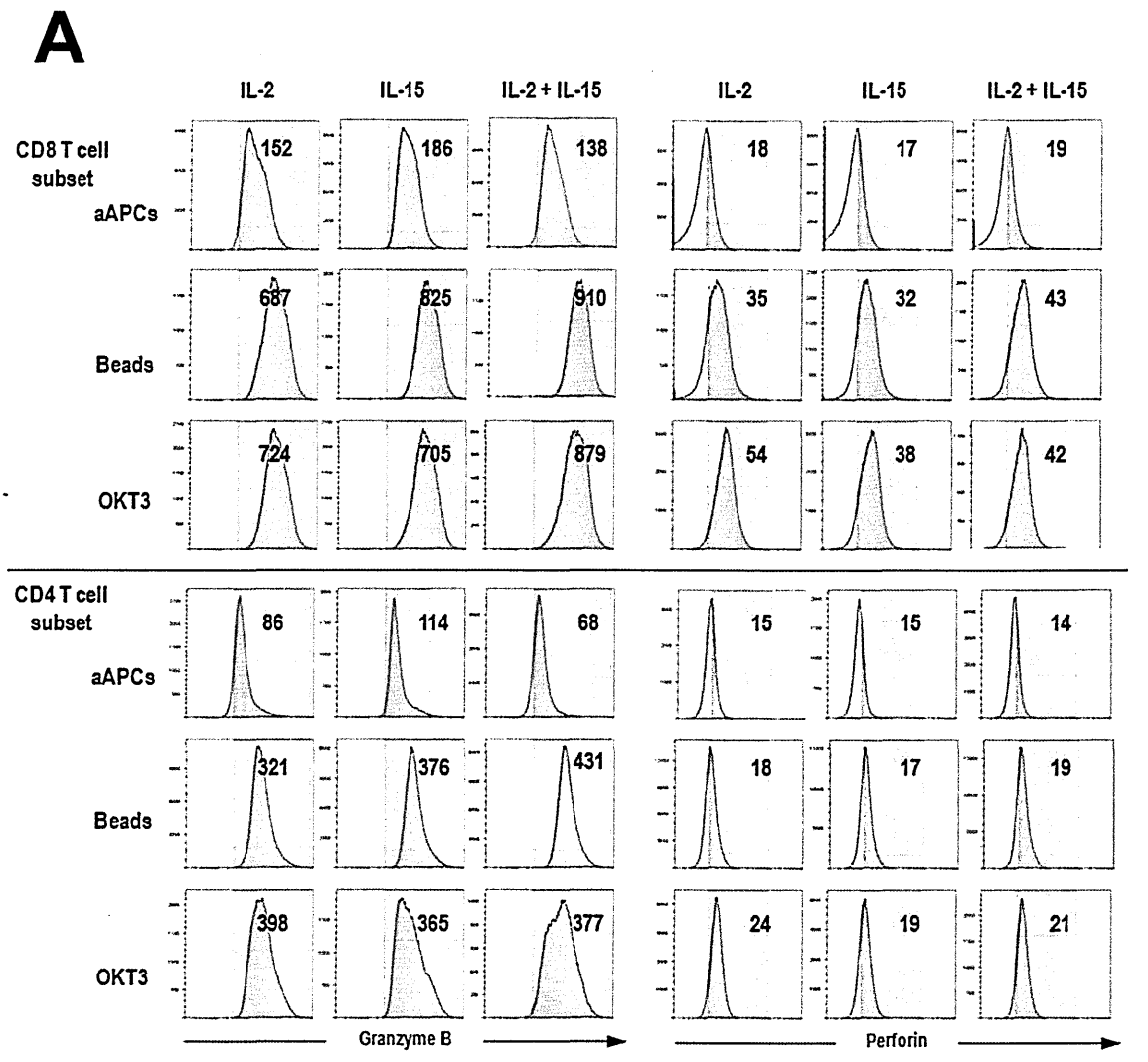
Figure 5.6 Phenotypic characteristics of polyclonal human T cells expanded with aAPC or other artificial systems.

After 14-day expansion in the presence of aAPC, microbeads or adherent anti-CD3 mAb plus HD IL-2 and IL-15, the maturation status of T cells was assessed by flow cytometry. (A) CD4+ and CD8+ T-cell frequencies are shown together with the frequencies of CD45RA+CD62L+, CD69+ and FOXP3+ cells in the two T-cell subsets. Results are representative of four independent experiments with different donors. Error bars indicate the standard deviation of the mean. Statistical analyses were performed using the two-tailed Student's *t* test (*: $p \leq 0.05$;**: $p \leq 0.01$; ***: $p \leq 0.001$). (B) A representative example of the T-cell immunophenotype after 14-day expansion with aAPC stimulation and HD IL-2 plus IL-15. Multiparametric FACS analysis was performed on CD4+ (CD4) and CD8+ (CD8) versus side scatter-gated cell populations. (C) Detailed analysis of maturation status of T cells expanded in the presence of aAPC or micreobeads. T_{CM} and naive T cells were defined, respectively, as the CD62L+CD45RA– and CD62L+CD45RA+ cell subsets in the CCR7+ gated population. T_{EM} and effector T cells were defined, respectively, as the CD62L–CD45RA– and CD62L–CD45RA+ cell subsets in the CCR7– gated population. The average values of three independent experiments and their standard deviations are reported (**: $p \leq 0.01$).

However, when CCR7 and CD27 expression was included in the analysis, aAPC showed a trend towards the preservation of an even higher immature phenotype (CCR7+ and CD27+) in stimulated CD4+ and CD8+ T cells, compared to microbeads (Figure 5.6C). In all experimental conditions, a large fraction of T cells showed a phenotype compatible with a highly activated status due to TCR engagement, as demonstrated by the wide expression of CD69 (Figure 5.6A-B).

Furthermore, amongst the artificial systems tested, aAPC determined the lowest up-regulation of Granzyme B expression (Figure 5.7A, left) and induced the lowest level of perforin (Figure 5.7A, right) in expanded CD8+ or CD4+ T cells. The reduced differentiating properties of aAPC were confirmed even at the analysis of the expression of perforin and Granzyme B in the CCR7+ and CCR7– subpopulations in both CD4+ and CD8+ cell subsets (Figure 5.7B). These observations suggest that T cells expanded in the presence of aAPC might exert a cytolytic function, since they express Granzyme B, but they require further activation, due to the limited induction of perforin. This phenotype is consistent with the high expression of CD62L and with the increased survival of T cells expanded with artificial APC. The concurrent adoptive transfer of naïve and activated cells, such as those derived by

aAPC stimulation, might be advantageous, since the expression of the homing adhesion molecule CD62L on the former should make them capable of trafficking through LN and becoming activated, whilst CD69+ and Granzyme B+ T cells could exert immediate effector functions.



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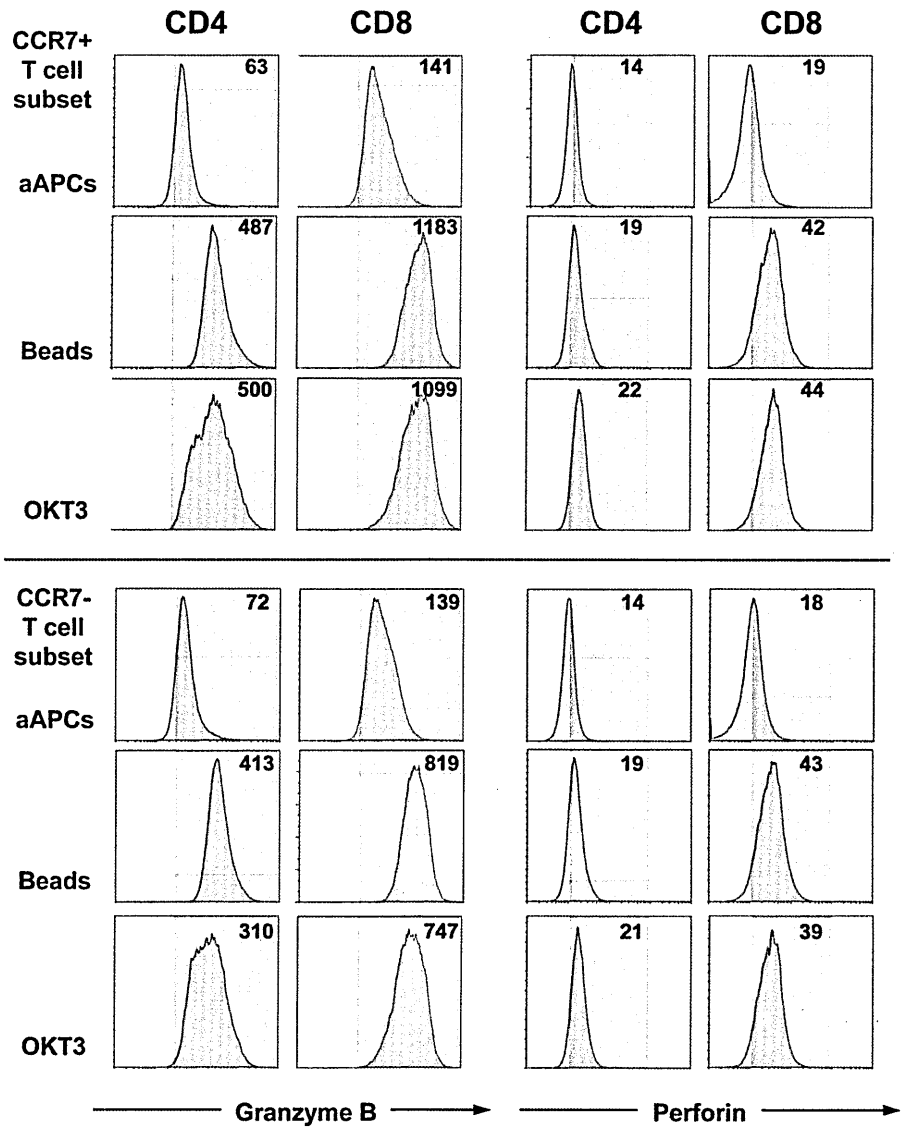


Figure 5.7 Flow cytometry analysis of Granzyme B and perforin expression in expanded T cells.
After 14-day expansion in the presence of aAPC, microbeads or adherent anti-CD3 mAb, the expression of Granzyme B and perforin was assessed by flow cytometry. (A) Histogram plot representation of intracellular expression of Granzyme B (left) and perforin (right) in CD3+CD8+ - (top), and CD3+CD4+ -gated T cells (bottom) stimulated in the presence of the indicated cytokines. (B) Histogram representation of Granzyme B (right) and perforin (left) expression in CCR7+ (top) or CCR7– (bottom) T cells from cultures expanded in the presence of HD Il-2 and IL-15 gated as CD3+CD8+ (CD8) and CD3+CD4+ (CD4). The MFI values of Granzyme B and perforin expression are reported in each plot.

Immunopheenotypic analysis of T cells after long-term expansion with aAPC showed a decrease of CD62L+ cell frequency and the parallel increase of CD45RA+CD62L– CCR7– effector cells with respect to the more shortly stimulated cultures (Table 5.1). In agreement with these results, after 27-day expansion, T cells down-regulated also CD25 expression, indicating the exhaustion of T cell activation (Table 5.1).

Similar results were obtained for microbead- or adherent anti-CD3 mAb-stimulated T cells (data not shown). Collectively, these observations suggested that 2 weeks of culture

was the optimal time window to expand a large amount of T cells with suitable immunophenotypic characteristics for adoptive immunotherapy.

Table 5.1 Immunophenotypic analyses of T cells expanded with complete aAPC or aAPC lacking anti-LFA-1 mAb.

	FACS analyses	Freshly isolated $CD3^+$T cells	aAPC stimulated $CD3^+$ T cells			
			+14 days		+27 days	
			+ anti-LFA-1 mAb	- anti-LFA-1 mAb	+ anti-LFA-1 mAb	- anti-LFA-1 mAb
CD4	CD4	43.57	26.42	25.89	9.18	6.99
	CD62L+	70.27	88.53	88.43	78.54	77.09
	CCR7+	60.09	31.37	15.89	28.71	18.01
	CCR7+CD45RA+CD62L+	72.38	24.90	13.30	23.17	16.03
	CCR7-CD45RA+CD62L-	10.51	5.08	6.30	13.40	15.27
	CD69+	0.45	10.47	7.82	6.72	4.67
	CD27+	89.49	84.14	80.69	86.75	81.15
	CD25+	7.96	46.65	41.75	16.00	15.69
	CD25-FOXP3+	0.62	0.41	0.35	1.58	2.17
	CD25+FOXP3+	2.40	1.10	0.65	0.78	0.69
CD8	CD8	20.81	58.85	58.06	75.98	80.78
	CD62L+	51.99	77.82	74.22	53.87	47.44
	CCR7+	41.27	17.69	8.56	28.24	11.22
	CCR7+CD45RA+CD62L+	75.60	15.60	6.90	20.45	8.98
	CCR7-CD45RA+CD62L-	50.13	18.65	22.57	37.35	48.34
	CD69+	1.14	7.99	24.68	29.66	31.23
	CD27+	77.14	85.04	71.11	79.31	70.41
	CD25+	0.66	23.15	13.67	2.52	1.96
	CD25-FOXP3+	0.13	1.97	1.42	1.49	3.40
	CD25+FOXP3+	0.21	1.04	0.52	0.26	0.48

Finally, the possibility to concurrently expand Tregs in culture after stimulation with the different artificial systems was evaluated. After 2-week expansion by aAPC, the frequencies of CD4+FOXP3+ and CD8+FOXP3+ T cell subsets were significantly lower compared to those induced by microbead- or adherent anti-CD3 mAb-stimulation (Figure 5.6 A-B). When Tregs were analysed, both CD25+ and CD25– FOXP3+ T cells were considered, given the recent evidence that FOXP3 expression can be found independently of CD25 (Walker, Kasprowicz et al. 2003).

5.4.1.4 Specific T-cell expansion with aAPC

Since aAPC were demonstrated to rapidly activate and expand polyclonal T lymphocytes, the possibility to efficiently also expand specific CTLs with this system was investigated. Lymphocytes from tumour-invaded LN of HLA-A*0201+ melanoma patients were activated in culture with the MART-1 peptide-loaded HLA-A*0201+ TAP-deficient T2 cell line. After 2 weeks of specific stimulation, the resulting T cell lines were expanded for 2 more weeks in the presence of the cognate antigen or standard aAPC, immobilized anti-CD3 mAb, or anti-CD3/-CD28 microbeads plus HD IL-2 and IL-15. The stimulation with the cognate antigen exhibited a limited expansion efficacy (Figure 5.8A) whilst maintaining a high percentage of MART-1 tetramer+ CD8+ T cells (73.5%). The highest efficiency in T-cell expansion was obtained using aAPC (Figure 5.8A), which also preserved T-cell viability better than did either microbeads or anti-CD3 mAb (Figure 5.8B).

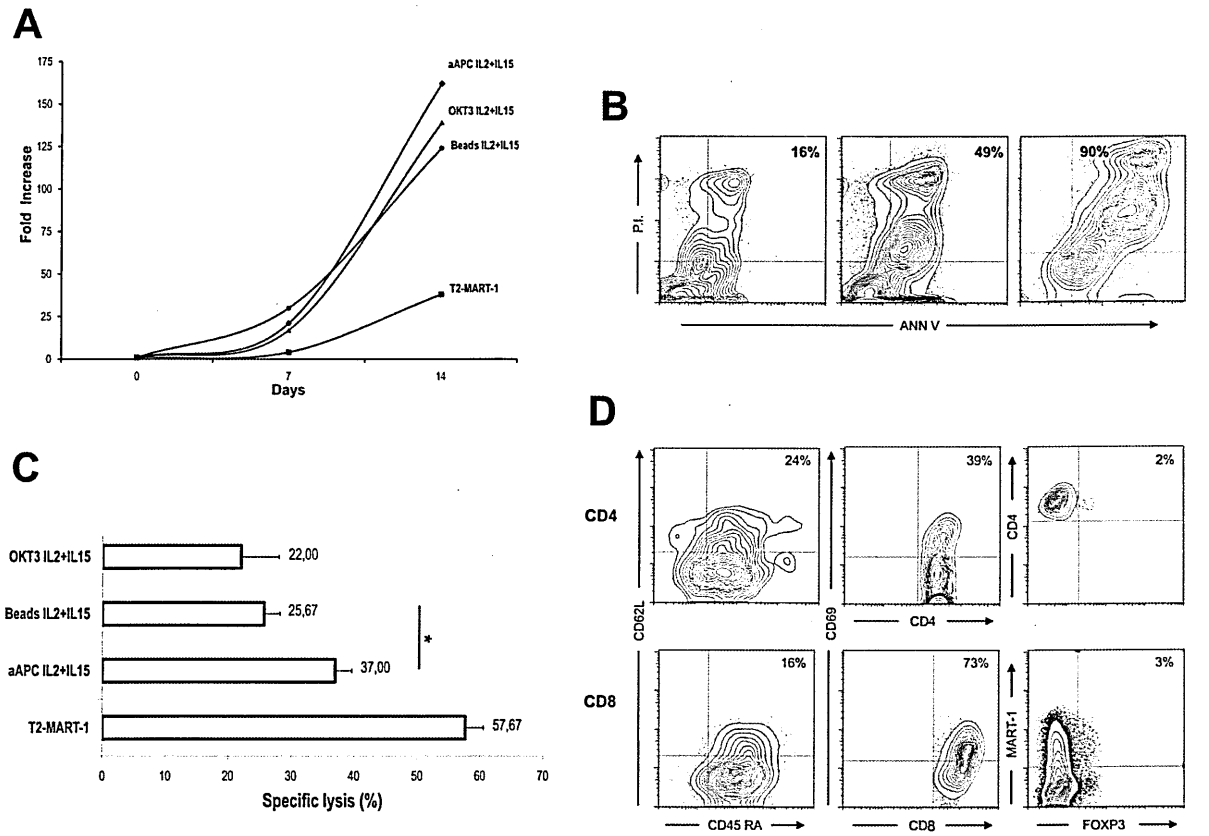


Figure 5.8 Expansion of anti-MART-1 human T lymphocytes.
(A) After 7 and 14 days of stimulation with anti-CD3/-CD28 microbeads or immobilized anti-CD3 mAb or aAPC in the presence of HD IL-2 and IL-15, MART-1 specific CD8+ T-cell cultures were counted using the Tripan blue exclusion test to measure their proliferation rate. X-axis, days in culture; y-axis, fold increase in cell number. (B) Representative flow cytometry analyses of apoptosis after 14-day expansion with aAPC (left), adherent anti-CD3 mAb (middle) or microbeads (right). X-axis: FITC-labelled ANN V; y-axis: PI. (C) Cytotoxicity of anti-MART-1 T-cell cultures after 14-day stimulation with aAPC or anti-CD3/-CD28 microbeads or immobilized anti-CD3 plus HD IL-2 and IL-15, or with MART-1 loaded T2 cell line (T2-MART-1) analysed by ^{51}Cr release assay using as target cells LCL pulsed with the specific MART-1 peptide. Specific lysis is shown as percentage of the unrelated lysis when target cells were unloaded or loaded with the unrelated Flu or HIV peptides. Results are representative of three independent experiments. Error bars indicate the standard deviation of the mean. Statistical analyses were performed using Student *t* test (*: $p \leq 0.05$). (D) Representative example of immunophenotype of anti-MART-1 T cell after 14-day expansion with aAPC. Flow cytometry analyses were performed on CD4+CD3+, CD4-CD3+ (CD8+ cells) and MART-1+CD4-CD3+ gated cell subpopulations. The expression of CD62L versus CD45RA, CD69 and of FOXP3 was evaluated to define, respectively, the maturation and activation level and Treg frequency in expanded cultures.

In addition, anti-MART-1 CD8+ T-cell cultures stimulated with aAPC showed an enhanced specific cytotoxic activity compared to microbead- or anti-CD3 mAb-stimulated T cells (Figure 5.8C). The greater ability of aAPC compared to microbeads to support CD8+ T-cell expansion in the polyclonal setting might partly explain the observed increased efficacy of aAPC to expand specific CD8+ T-cell cultures with high specific cytotoxicity (Figure 5.8C).

Immunophenotypic analysis of MART-1 T cell cultures expanded with aAPC revealed that both CD8+ and CD4+ T cells still expressed a relatively high levels of the LN homing molecule CD62L, whilst displaying a broad CD69 positivity. These results indicate that the stimulation provided by aAPC is able to activate specific CTL as efficiently as polyclonal T cells (Figure 5.8D). In agreement with previous findings, the frequency of FOXP3+ T cells did not increase either in total CD8+, anti-MART-1 CD8+, or in CD4+ T cell subsets after 14 days of culture with aAPC (Figure 5.8D).

The comparison of the maturation status of MART-1 specific T-cell cultures before and after 2-week stimulation with aAPC or microbeads, showed the maintenance of an effector memory phenotype of T cells induced by the previous TCR stimulation with the cognate antigen (Figure 5.9, bottom vs. top). Interestingly, aAPC-expanded T cell cultures showed a higher frequency of MART-1 specific CD8+ T cells as compared to the microbead-expanded cultures (Figure 5.9, bottom left vs. right).

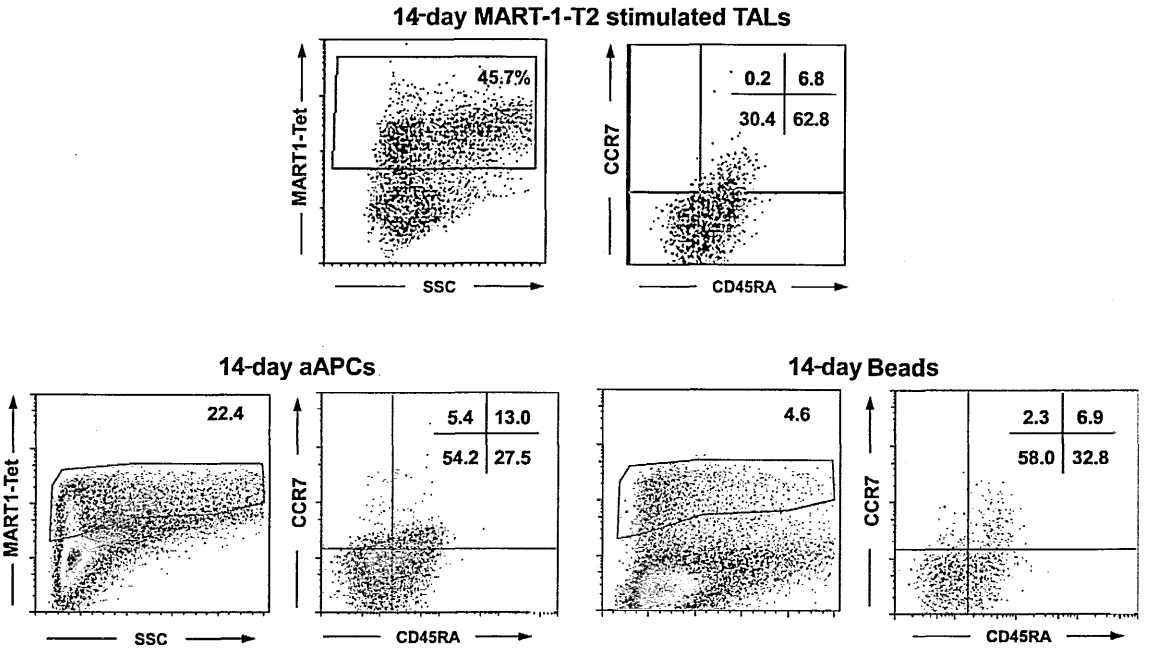


Figure 5.9 Immunophenotype of aAPC-expanded MART-1-specific T cells.
T cells isolated from melanoma infiltrating LNs were cultured for 2 weeks in the presence of MART-1-loaded T2 cells (TALs). The frequency of MART-1-specific T cells was then detected using MART-1 tetramers (MART-1-tet) and revealed by flow cytometry in the CD3+CD4– gated T-cell subset (top left). The maturation status of these cells was evaluated by flow cytometry analysis of CCR7 and CD45RA expression in the CD3+CD4–MART-1-tet+-gated T cell subset (top right). The same parameters were assessed after additional 2 weeks of aAPC (bottom left) or microbead (bottom right) stimulation in the presence of HD IL-2 and IL-15.

5.4.1.5 Optimization of aAPC-based system for T cell expansion

In order to verify the feasibility of the procedure in the light of large–scale clinical application, several experiments were performed. First, the results obtained by comparing aAPC with anti-CD3/-CD28 microbeads for research use were corroborated by a further comparison with the clinical grade ones (Dynabeads® ClinExVivo CD3/CD28).

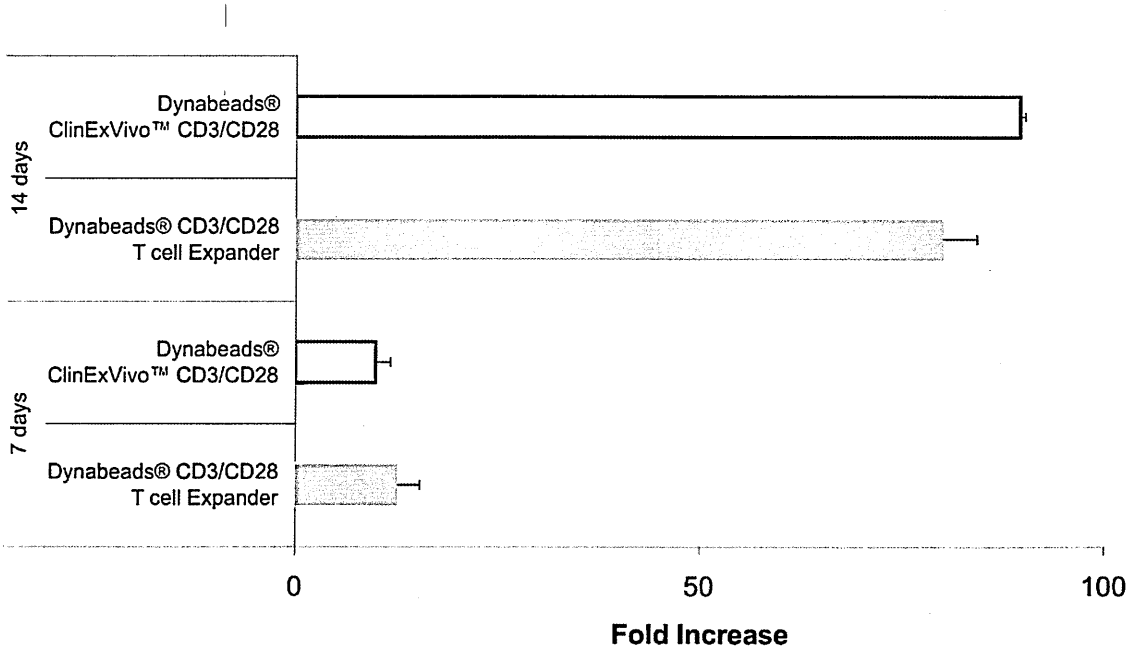


Figure 5.10 T-cell expansion efficiency of anti-CD28/-CD3 microbeads developed for research and clinical use. Fold increase in ANN V-/PI- viable T cell number calculated on the basis of the flow cytometry analysis of apoptosis and the Trian blu exclusion test performed on polyclonal T-cell cultures after 7 and 14 days from a single stimulation with the anti-CD28/-CD3 microbeads for research (Dynabeads® CD3/CD28 T Cell Expander) or clinical use (Dynabeads® ClinExVivo CD3/CD28), in the presence of HD IL-2 and IL-15.

The stimulation with the research and the clinical version of anti-CD3/CD28 microbeads provided comparable T-cell expansion, definitely proving the advantages of aAPC over this system (Figure 5.10). In order to evaluate the possibility of using aAPC in compliance with GMP requirements, the persistence of aAPC components in expanded cultures was assessed by flow cytometry, after both 7 and 14 days from stimulation. To this aim, a regression line was derived to evaluate the sensitivity of mouse mAb detection on a fixed T-cell number ($5x10^4$) on the basis of MFI values using a FITC-labelled anti-mouse IgG Ab (Figure 5.11A). Using this assay, the amount of mouse anti-human mAbs of aAPC MDs that still interact with T cells after 7 or 14 days of culture could be thus extrapolated (Figure 5.11B). Both these values (0.08 and 0.04 pmol found at days 7 and 14, respectively, Figure 5.11A) were at the lowest left end of the regression line, indicating a reduction of more than two logs in mouse mAb concentration on human T cells compared to the starting amount (represented by the highest value on the right of the titration curve).

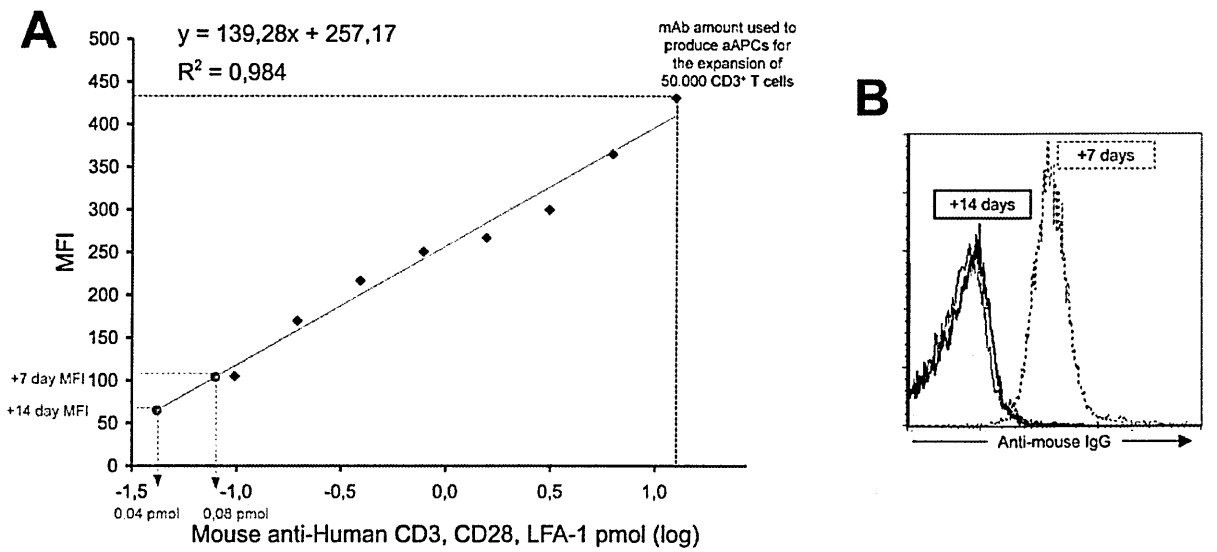
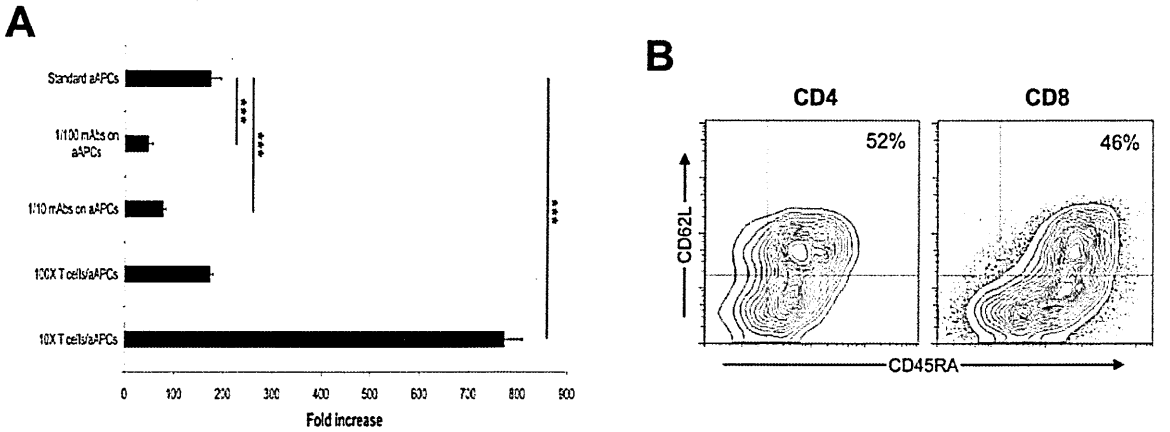


Figure 5.11 Persistence of aAPC in T-cell cultures.

(A) Regression line obtained by plotting MFI values (y-axis) of T cells pre-incubated with decreasing concentrations (x-axis) of anti-CD3, anti-CD28, anti-LFA-1 mAb and stained with the proper secondary FITC-labelled Ab. Regression analysis of the data yielded a linear relationship between 0.1 and 10 pmol (described by the indicated equation). (B) Flow cytometry analysis of aAPC persistence after 7- (dotted line) and 14-day (black line) expansion. Unstimulated T cells stained with FITC-labelled anti-mouse IgG (grey filled histogram) were used as a control. MFI revealed at days 7 and 14 of culture are shown in A on the titration curve as grey dots.

Finally, two alternative strategies were investigated to reduce the cost of aAPC generation for large-scale application, whilst at the same time preserving their T-cell expansion efficiency. Initially the concentration of mAbs per aAPC was decreased by 1 or 2 logs. However, these aAPC were less efficient than the standard ones in expanding T cells when the APC/T-cell ratio was unchanged (Figure 5.12). The observed activity loss might most likely be derived from the reduced probability to obtain MDs that were completely saturated with biotionylated mAb after this modification. Therefore, the possibility of decreasing the aAPC/T-cell ratio for T-cell expansion was investigated. Remarkably, an efficient T-cell expansion was achieved when the aAPC/T-cell ratio was reduced by 1 or 2 logs (i.e. by keeping the amount of aAPC constant and increasing the starting T cell number by 10-100 fold, Figure 5.12A). Unexpectedly, an increase of 10 fold of the T-cell numbers per aAPC boosted T-cell proliferation even more than the standard condition (Figure 5.12A). This suggested that the strength of T-cell stimulation is critical for the overall expansion yield and indicates the potential advantages of this system for the stimulation of large-scale cultures in which T cells may amplify the potent aAPC stimulation through direct interactions and/or the release of soluble mediators.



C

aAPCs/ T cell ratio	Starting culture volume (L)	Final culture volume (L)	aAPC dose fold increase
1/5x10^4	3.268	50	1274
1/5x10^5	0.031	20	31
1/5x10^6	0.051	8	13

Figure 5.12 Optimization of aAPC protocol for T-cell expansion.
(A) Fold increase in T-cell number after stimulation with standard aAPC, aAPC with 1- (1/10 mAbs on aAPC) or 2-log (1/100 mAbs on aAPC) lower mAb concentration, or with 1 (10X T cells/aAPC) or 2-log (100X T cells/aAPC) reduced aAPC/T-cell ratio in culture. Freshly isolated polyclonal CD3+ T cells were stimulated under these conditions in the presence of HD IL-2 plus IL-15. Cell counts were assessed using the Tripan blue exclusion test on days 7 and 14 after stimulation. Results are representative of three independent experiments with different donors. Error bars indicate the standard deviations of the mean (***: $p \leq 0.001$). (B) Representative example of CD45RA and CD62L expression after 14-day stimulation in the presence of 1 or 2-log reduced aAPC/T-cell ratio in cultures. (C) Estimation of the amount of aAPC and the culture volumes required for the expansion of 1x10^{10} cells (target T cell number) using different aAPC/ T cell ratios.

In these expansion conditions, the potential capability of *in-vivo* persistence, based on CD62L expression, was preserved (Figure 5.12B). In addition, using a 1- or 2-log decreased aAPC dose/ T cell ratio, 10 billion anti-tumour T cells could be generated in a reduced volume with a limited cost, making this procedure suitable for clinical application (Figure 5.12C).

5.4.2 Functional role of OX40 expression in lymphoma microenvironment: towards OX40 agonist-based anticancer immunotherapy

5.4.2.1 OX40 expression and immunosuppressive function of Treg infiltrating indolent NHL

As shown in Chapters 2 and 3, the modulation of Treg frequency in the PB and at the tumour site of vaccinated patients with indolent NHL played a crucial role in dictating patients' outcome. Given the recent findings suggest an important involvement of OX40 signalling in the control of tolerance induced by Tregs in preclinical models (Valzasina, Guiducci et al. 2005; Piconese, Valzasina et al. 2008; Colombo and Piconese 2007), OX40 expression on human Tregs as well as its functional role in the indolent NHL microenvironment was investigated. Previous results described in Chapter 2 (Paragraph 2.4.3.1) demonstrated a progressive increase in CD4+CD25+FOXP3+ Treg frequency from PB towards the tumour sites in NHL patients. Interestingly, OX40 was more widely expressed on PB Tregs from lymphoma patients compared to healthy donors, and its expression was even more up-regulated on tumour-infiltrating Tregs (Figure 5.13 A-B).

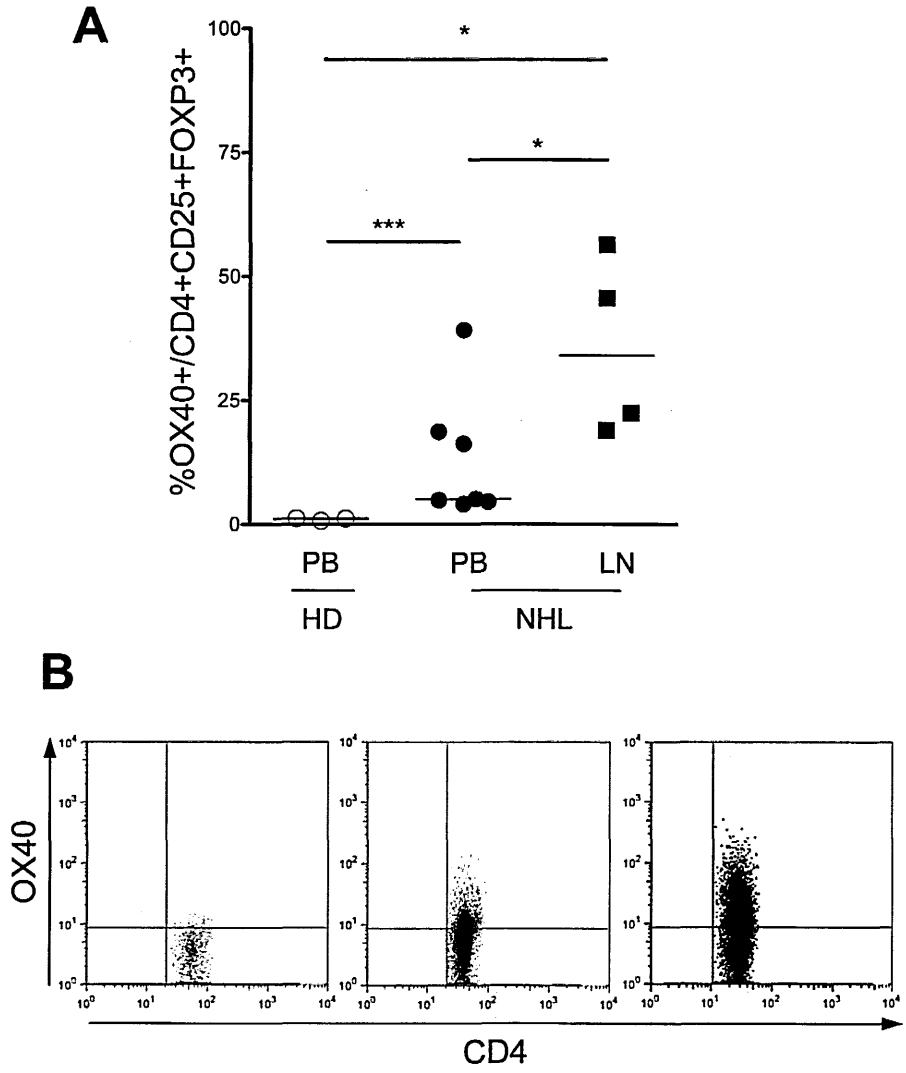


Figure 5.13 OX40 expression on donors' or NHL patients' Tregs.

(A) Flow cytometry analysis of OX40+ Treg frequency in healthy donor (HD) PB or in PB and malignant LN from patients with B-NHL. (B) Representative examples of OX40 expression in CD4+CD25+FOXP3+ -gated cells in donors' (left), NHL patients' PB (middle) or malignant LN (right). *: p≤0.05; **: ***: p≤0.001.

To verify that the phenotypically identified CD4+CD25+FOXP3+ T cells were truly Tregs, their ability to suppress the proliferation of CD4+CD25- Teffs stimulated with PHA was tested *in vitro* using a standard proliferation assay. CD4+CD25+ Tregs and CD4+CD25- Teffs were purified from tumour cell suspensions obtained from pathologic LN. As shown in Figure 5.14, after a 5-day incubation with PHA, CFSE-labelled Teffs were highly proliferating, with five generations being observed (top left). Ninety-four percent of these cells expressed OX40 (top right). When Tregs were added in culture, the Teffs failed to respond to PHA stimulation (Figure 5.14, bottom left), even though they could still up-regulate OX40 expression (Figure 5.14, bottom right). Interestingly, the CFSE- Tregs became fully OX40-positive upon PHA stimulation (Figure 5.14, bottom right). These results indicate that a positive TCR stimulation might be responsible for the induction of OX40 expression in either of the T-cell subsets, which in turn can be associated with proliferation in case of Teffs or the immunesuppressive activity in case of Tregs.

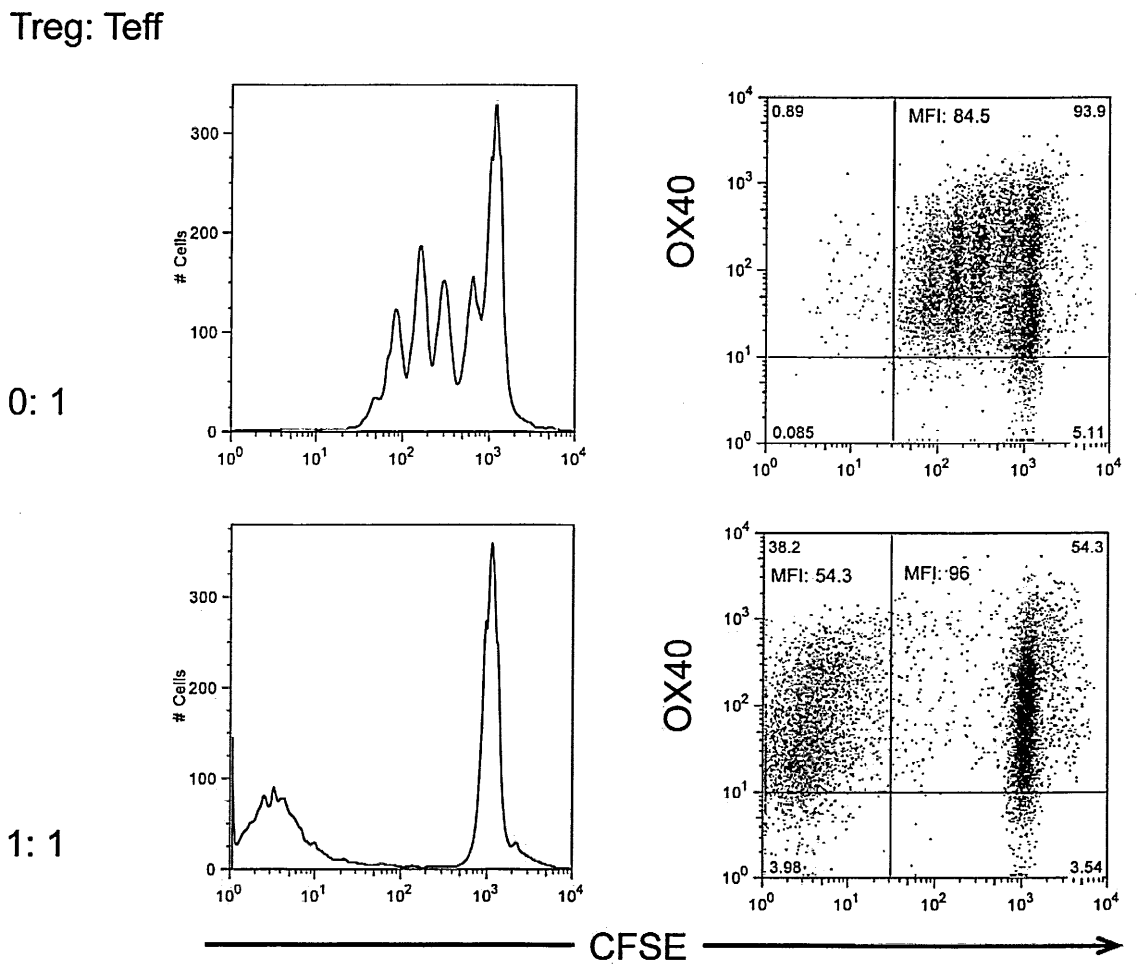


Figure 5.14 Immunosuppressive activity of Tregs infiltrating NHL lesions.
CFSE-labelled CD4+CD25low/- Teffs were purified from primary NHL biopsies and cultured in the presence of PHA alone (top) or with CD4+CD25high Tregs (bottom) isolated from the same tumour at the indicated ratio. A representative flow cytometry analysis of CFSE dilution in Teffs (left) as well as OX40 expression (right) in CFSE+ Teffs and CFSE- Tregs after 5-day stimulation with PHA is shown. MFI of OX40 staining is reported in the dot plots.

OX40 expression was then studied in the Tregs of patients before and after vaccination. Interestingly, in Rs, the reduction of Treg frequency in both PB and LN was

paralleled by their down-modulation of OX40 expression (Figure 5.15 top left and bottom, for representative examples). In case of disease reactivation, Tregs that reappeared in the pathologic LN showed a concurrent up-regulation of OX40 expression (Figure 5.15 bottom). In NRs no significant modulations of either OX40+ Tregs or OX40+ Teffs were detected after vaccination (Figure 5.15 top right, for a representative example). These observations suggest that a clinical response may be favoured by the reduction and/or inactivation of immunosuppressive cells after vaccination.

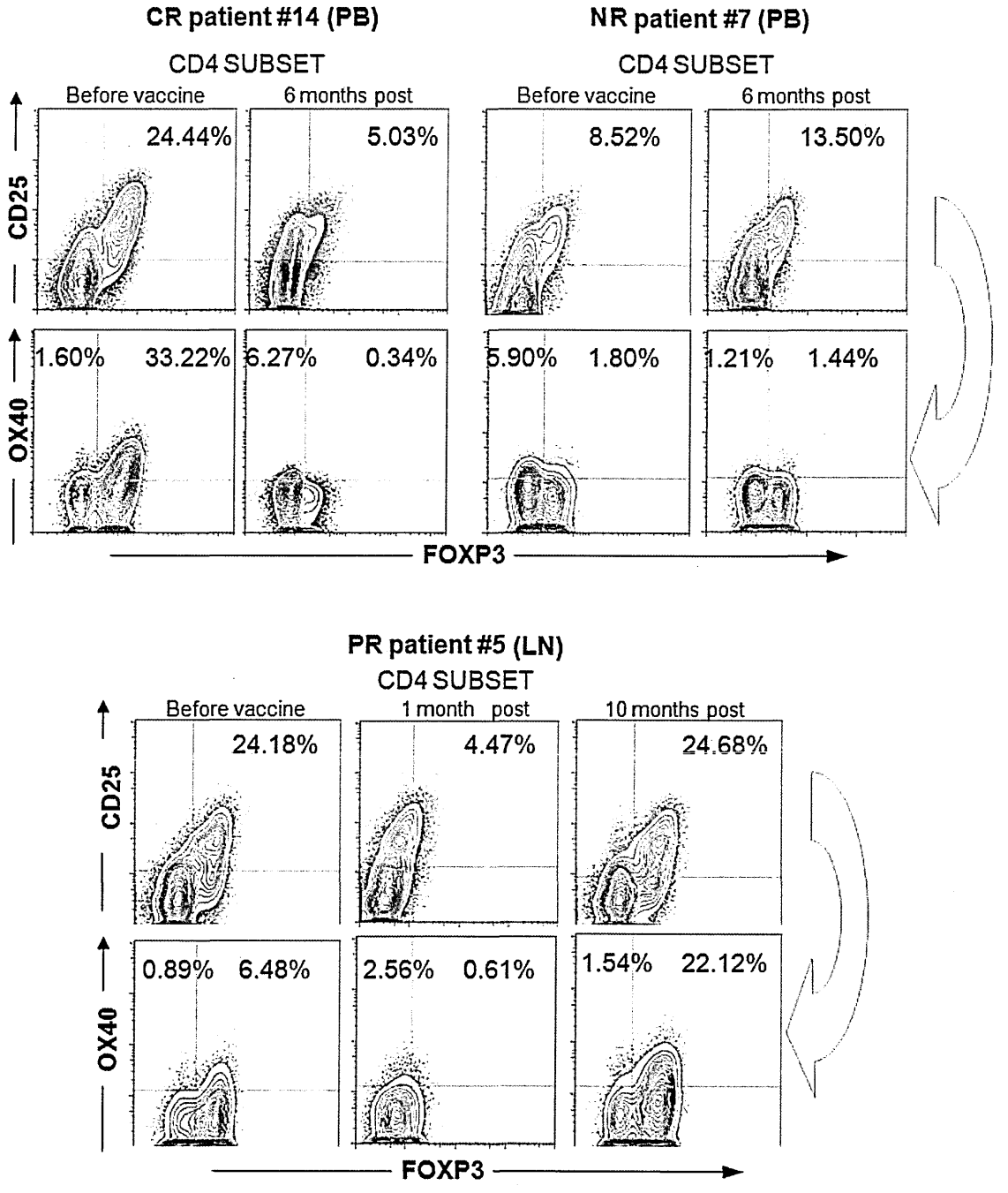


Figure 5.15 OX40 expression in Tregs and Teffs before and after vaccination.
Flow cytometry analysis of CD25 vs. FOXP3 expression in CD4+ -gated T cells and of OX40 vs. FOXP3 in CD4+CD25+ -gated T cells in pre- and post-vaccine PB samples of one CR and one NR and LN biopsies of one PR harvested before vaccination, at time of response (6 month post) and after disease progression (10 months post).

5.4.2.2 OX40 functional role in NHL microenvironment

The modulation of OX40 expression on T cells according to their activation status and the reduction of Tregs together with their down-regulation of OX40 in Rs after DC-vaccination led to the investigation concerning the functional role of OX40 on these cells in the lymphoma microenvironment.

Given the recent finding that OX40 triggering on murine Tregs by an agonist Ab can inhibit their immunosuppressive activity (Valzasina, Guiducci et al. 2005; Piconese, Valzasina et al. 2008; Colombo and Piconese 2007), the possibility that human Tregs could be inactivate through OX40 stimulation was investigated as an alternative way to exploit OX40-OX40L pathway for anticancer purposes. If true, the resultant dual effects of inhibiting Treg and enhancing Teff functions upon OX40 triggering would be expected to drive the re-balancing of tumour immune-surveillance towards a therapeutic effect. Unfortunately, the lack of commercially available agonist anti-OX40 Abs has greatly hampered this study. The use of a trimeric OX40L recombinant protein, whilst confirming to stimulate survival and proliferation of human Teffs (Figure 5.16 A), determined only a modest inhibition of Treg-mediated suppression of Teff proliferation in standard CFSE proliferation assays (Fig. 5.16 B).

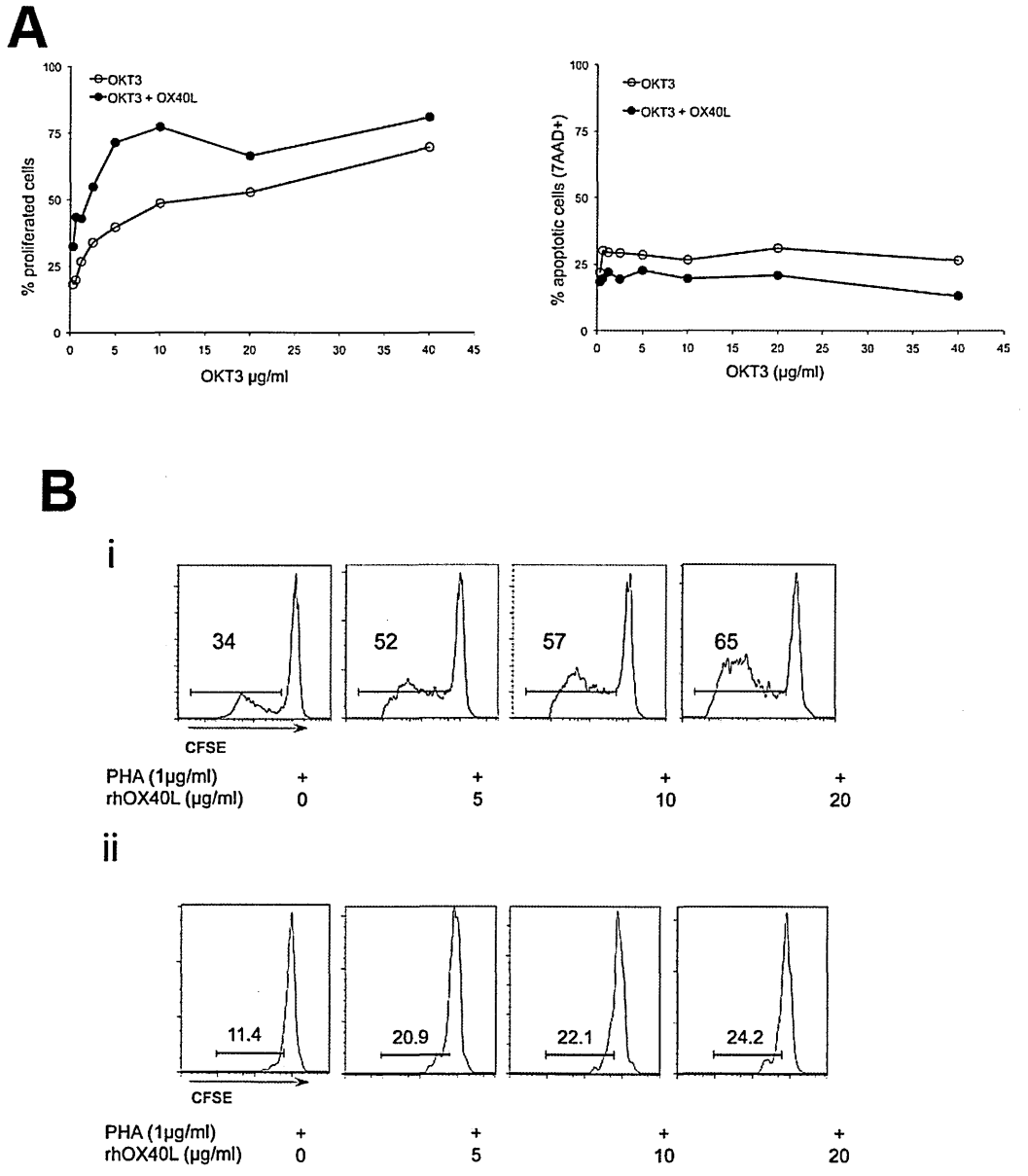


Figure 5.16 Effects of OX40 triggering on Teffs and Tregs by recombinant human OX40L fusion protein.
CD4+CD25low/- Teffs and CD4+CD25high Tregs were purified from PBMC or lymphoma biopsies and used in standard proliferation assay. (A) PHA pre-activated donors' Teffs were labelled with CFSE and cultured with escalating dose of immobilized anti-human CD3 mAb (OKT3 clone) in the presence (filled circles) or not (empty circles) of 10μg/ml soluble recombinant human OX40L fusion protein. After 4 days, flow cytometry analyses of CFSE dilution and 7-AAD staining were performed to evaluate, respectively, proliferation (left) and cell-death (right) in cultures. (B) CFSE-labelled Teffs purified from lymphoma cell suspension were cultured alone (i) or with autologous Tregs (ii) in the presence of PHA. After 24 hours, OX40L recombinant fusion protein was added at the indicated concentration. Teff proliferation was monitored by flow cytometry analysis of CFSE dilution in CFSE+-gated cells.

Therefore, the study was directed towards the analysis of the potential function of OX40 and OX40L expression in the lymphoma microenvironment. The modulation of these antigens was thus investigated in B- and T-cell subsets within the cell suspensions obtained from normal reactive and malignant LN, before and after stimulation with PHA. Remarkably, upon activation, not only T cells, but also CD19+ B cells, up-regulated both OX40 and OX40L expression (Figure 5.17 Ai and Bi). These effects were even stronger in the pathologic compared to the reactive LNs (Figure 5.17 Bi *vs.* Ai), suggesting that OX40 and OX40L up-regulation on lymphoma cells, as a consequence of TIL activation, may provide pro-survival signals through the co-stimulation of these molecules on tumour cells.

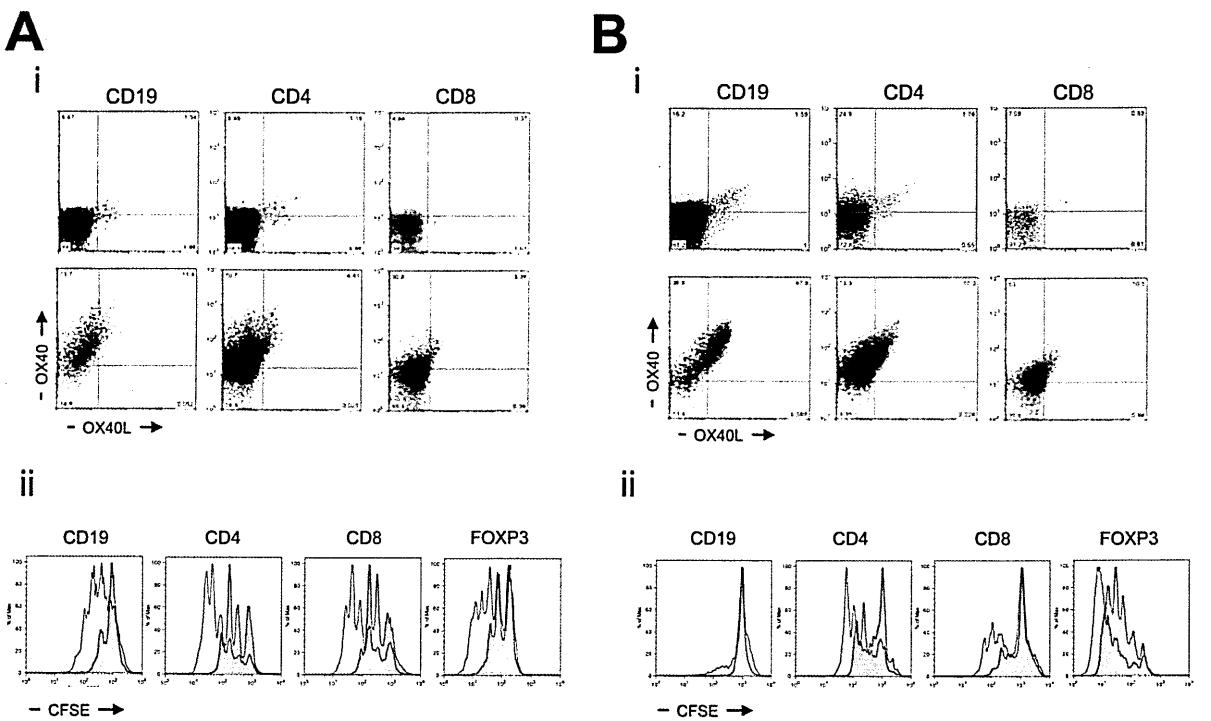


Figure 5.17 OX40-OX40L co-stimulation in the lymphoma microenvironment.
Normal reactive (A) and malignant (B) LN cell suspension were labelled with CFSE and cultured in the presence of 1μg/ml PHA. Before (i, top) and after 4-day (i, bottom) stimulation, OX40 and OX40L expression was evaluated by flow cytometry in CD19+-, CD4+- and CD8+-gated cell subpopulations. Flow cytometry analysis of CFSE dilution (ii) was performed after 4-day culture in the presence (filled grey histogram) or not (black line histogram) of 5μg/ml soluble anti-OX40 neutralizing mAb.

Accordingly, the addition of an OX40 neutralizing mAb impaired PHA-induced proliferation in all cell subsets analysed in both normal and malignant LN, including B cells (Figure 5.17 Aii and Bii, respectively). This finding led to the analysis of OX40 and OX40L expression on B-lymphoma cells. A panel of human B-NHL cell lines was studied by flow

cytometry. PBMCs pooled from 6 healthy donors were analysed in parallel as control. Whilst OX40L was not significantly detected in any samples analysed, 30 to 75% of cells in NHL cell line cultures expressed OX40 (Figure 5.18A). Interestingly, compared to normal B cells, NHL cell lines showed higher level of OX40 expression, as revealed by the comparison of the MFI values (Figure 5.18B).

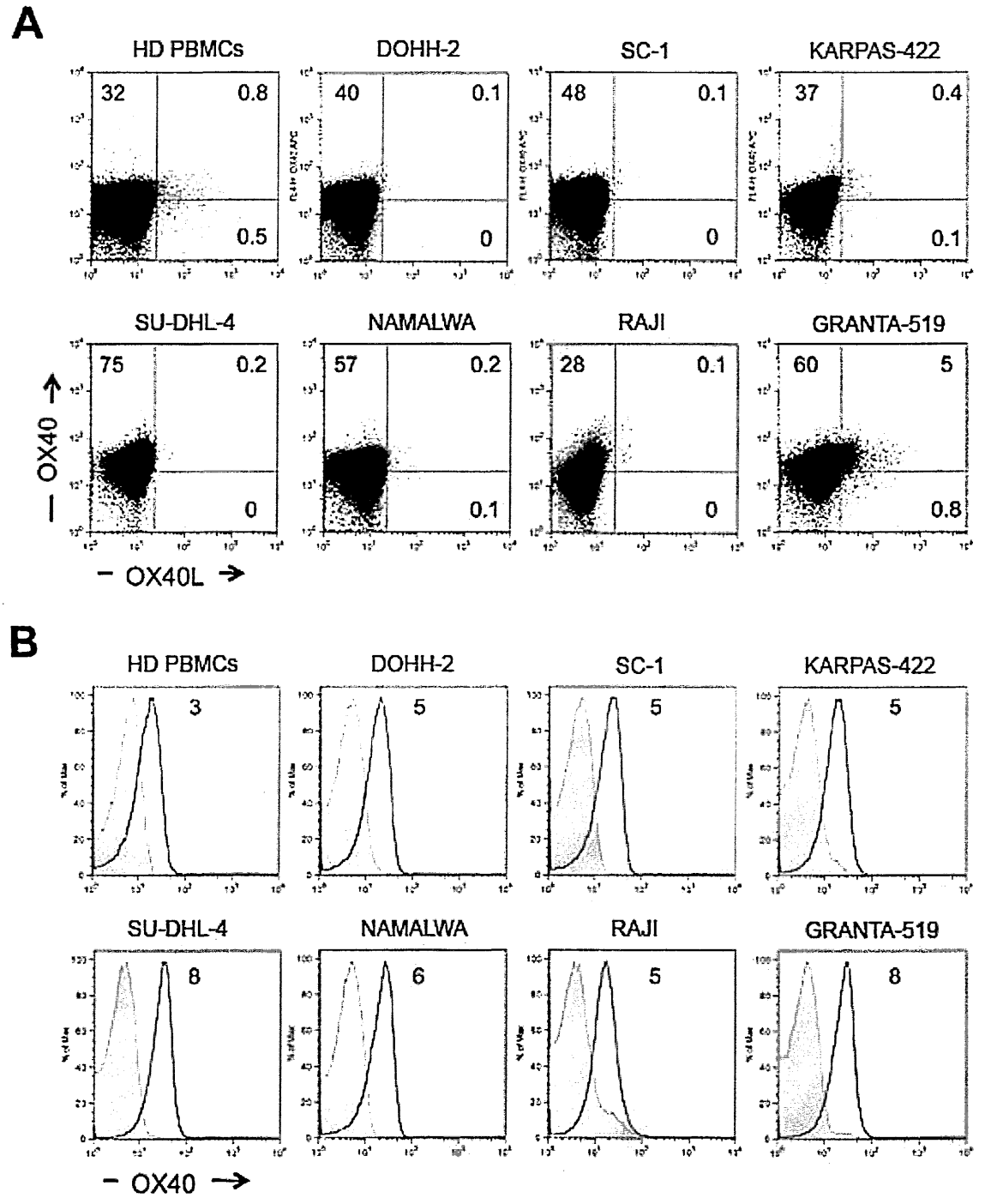


Figure 5.18 Analysis of OX40 and OX40L expression in NHL cell lines and donors' PBMCs.
(A) Flow cytometry analysis of OX40L and OX40 in CD20+ -gated cell subset of pooled PBMCs from 6 healthy donors (HD PBMCs), or the following NHL cell lines: DOHH-2, SC-1, KARPAS-422, SU-DHL-4, NAMALWA, RAJI, GRANTA-519. Cell frequency in the OX40L (x-axis) *vs.* OX40 (y-axis) subsets are showed in each plot. (B) Histogram plot of OX40 expression in the same samples showed in A. Relative OX40 MFI value obtained by the ratio between stained (black line) *vs.* isotype control (filled grey) MFI is indicated for each plot.

5.5 Discussion

The Chapter describes the findings obtained from the study of a novel aAPC-based system for the polyclonal expansion of T cells for ACT and the functional role of OX40 co-stimulation in the lymphoma microenvironment. These represent two different ways to enhance, respectively, the afferent and the effector phases of an anti-tumour immune response in combinatorial immunotherapeutic approaches.

Results of T-cell stimulation through aAPC (produced by preclustering anti-CD3, -CD28 and –LFA-1 mAbs on liposome structures to resemble the physiological lipid rafts of APC plasma membranes) were as follows: (i) the generation of large amounts of T cells starting from polyclonal CD3+ T lymphocytes (ii) that displayed the highest cell viability in culture compared to the other commercial artificial systems, and (iii) a naïve or effector phenotype, (iv) without showing the increase of CD25highFOXP3+ Treg frequency; (v) the expansion of functionally competent anti-MART-1 CD8+ T cells; and (vi) the feasibility to apply this system large-scale settings.

Recent reports have indicated that pre-clustering MHC-peptide complexes in MDs on artificial lipid membrane positively affects the efficiency of immune synapse formation and the ensuing T-cell activation (Anderson, Hiltbold et al. 2000; Vogt, Spindeldreher et al. 2002). Building upon these data, the liposome-based aAPC previously described by Albani and colleagues (Prakken, Wauben et al. 2000; Giannoni, Barnett et al. 2005), were modified by changing the MD constitution through the replacement of the HLA-peptide complex with an anti-CD3 mAb, combined with the addition of an anti-LFA-1 mAb, thus permitting a more efficient stabilization of aAPC-T cell interactions. These modifications allowed for the generation of novel aAPC capable to efficiently stimulate and expand T cells irrespective to their antigen specificity. Given the crucial role of CD4+ T-cell helper functions for adoptively transferred CTL activity and their *in-vivo* persistence, and the advantages of polyclonal, over tumour-antigen specific, CD8+ T cells for ACT (Antony, Piccirillo, et al. 2005; Corthay, Skovseth et al. 2005; Perez-Diez, Joncker et al. 2007; Hunder, Wallen et al. 2008), most artificial systems have been developed to express only the relevant molecules for an aspecific T cell stimulation. Although several artificial system have been produced (Maus, Thomas, et al. 2002) (Oelke and Schneck 2004; Derdak, Kueng et al. 2006; Suhoski, Golovina et al. 2007), only anti-CD3/-CD28 mAb immunomagnetic microbeads (Laport, Levine et al. 2003; Thompson, Figlin et al. 2003) and the stimulation with anti-CD3 mAb immobilized on culture plates have been generated under GMP requirements for clinical use. Compared to the original methods, based on the use of natural APC (nAPC), such as DCs or virally infected B cells, these technologies have been shown to expand larger numbers of T cells in a reduced time with the advantage that they are produced following standard protocols, and thus display reproducible immunostimulatory functions. However, compared to these artificial systems, liposome-based aAPC have additional advantages of including, amongst T-cell triggering molecules, an anti-LFA-1 mAb, which provide signals that physiologically are mediated by one of the most relevant adhesion molecules (ICAM-1), and a fluid membrane rather than a rigid scaffold, which allows MDs to freely cluster and orient towards T cells for the immunological synapse formation. Remarkably, after 2 weeks of stimulation in the presence of IL-2 and IL-15, T cells expanded with aAPC showed significantly lower levels of apoptosis and/or necrosis compared to those cultured with microbeads or an adherent anti-CD3 mAb. This result is of great relevance for the practical development of aAPC-based adoptive immunotherapy protocols. Furthermore, in contrast to microbeads and the adherent anti-CD3 mAb system, which boosted T-cell expansion towards exhaustion (as indicated by the high levels of cell-death in culture), T cells generated after aAPC stimulation were, in part, acutely activated (becoming CD69+) to exert immediate effector functions. They also, in part, expressed the specific homing molecules CD62L and CCR7, thus retaining the potential to traffic through the LNs, be re-

stimulated *in-vivo* and thus persist in the memory compartment (Gattinoni, Klebanoff et al. 2005). Potential explanations for these results include the ability of aAPC-mediated TCR triggering to expand naïve T cells, and to induce, in the presence of γ-chain cytokines, central and effector memory T-cell differentiation (Geginat, Sallusto et al. 2001; Geginat, Lanzavecchia et al. 2003). These characteristics make aAPC-expanded T cells more suitable for ACT compared to those generated in the presence of the other clinical grade artificial expansion systems. Additional support for this arises from multiple studies showing the inverse correlation between the maturation level of *in-vitro* stimulated T lymphocytes and their *in-vivo* anti-tumour activity and persistence potential (Gattinoni, Klebanoff, et al. 2005; Zhou, Shen et al. 2005; Yee, Thompson, et al. 2002). In addition, aAPC displayed the greatest capability to sustain CD8+ T-cell proliferation, whilst providing no increase in Treg frequency. This is in sharp contrast to the other artificial systems tested, further indicating the potential improvement of using aAPC to expand T cells for anticancer adoptive immunotherapy. Furthermore, when tested for the expansion of pre-enriched anti-MART-1 CD8+ T cells, aAPC displayed similar properties, with the additional advantage of being able to efficiently re-stimulate these cells whilst preserving their antigen specificity. Finally, the possibility of stimulating higher amount of T cells per aAPC in a reduced culture volume compared to the standard condition, without affecting T cell expansion efficiency, demonstrated the potential to scale up this approach for clinical purposes. In particular, aAPC may find an application for the aspecific *ex-vivo* expansion of the complete T-cell repertoire of cancer patients for ACT, with the aim to exogenously increase the frequency of TAA-specific lymphocytes that have often low antigen avidity and are usually under-represented or not sufficiently amplified by active vaccination (Palucka, Ueno et al. 2007; Rosenberg, Yang et al. 2004). In addition, in the case of highly immunogenic tumours, for which TAAs are known and the related peptides available, aAPC can be exploited to boost the expansion of previously enriched antigen-specific CD8+ T cells without any limitations due to HLA compatibility (Rosenberg, Restifo et al. 2008).

Experience from past clinical trials have shown that for the immunotherapy of human cancer it is unlikely that one strategy will be sufficient to reverse the tumour immune evasion phase towards the elimination of the tumour, mainly in those patients with conspicuous disease (Koos, Josephs et al. 2010). Of particular importance is the *in-vivo* generation, through active vaccination or ACT, of anti-tumour T cells with appropriate antigen specificity, which are available in numbers compatible with the induction of an effective anti-tumour immune response. Such cells must persist over time in order to break tumour immune tolerance to achieve therapeutically relevant effects. The strong dependency of T-cell survival and memory development upon OX40-OX40L interactions, and their concurrent ability to augment NK and NKT cell function whilst reversing Treg-mediated tolerance (Croft 2010), make OX40 an attractive target for adjuvant immunotherapy to enhance vaccine- and/or ACT-mediated anticancer protection. In addition, as the expression of OX40 is mainly restricted to antigen-activated immune cells, its targeting may results in a relatively specific immune response being less likely to induce autoimmune side effects when compared to that of more-broadly expressed co-stimulatory molecules (Croft 2010; Ishii, Takahashi et al. 2010). Preclinical studies have already demonstrated that OX40 agonists can augment protection against several solid tumours (Weinberg, Rivera et al. 2000) (Croft 2009). However, because the potential anti-tolerogenic effect of OX40 triggering on mouse Tregs has only recently been recognized (Valzasina, Guiducci et al. 2005; Piconese, Valzasina et al. 2008; Colombo and Piconese 2007), its contribution to these results remains unclear.

The observed significant increase of OX40 expression on lymphoma-infiltrating versus PB-derived Tregs and its up-regulation upon TCR triggering on these cells in association with their suppressive activity may suggest a crucial role for OX40+ Tregs in the tumour microenvironment. In this situation, indeed, the pro-inflammatory stimuli that could serve

as feeder milieu for lymphoma need to be kept in check to avoid the initiation of an anti-tumour immune response (Gribben 2010). Accordingly, the reduction of OX40+ Tregs with the clinical remission of vaccinated patients and their reappearance within the tumour at relapse further highlight a potential symbiotic relationship between a growing lymphoma and this cell subset. Interestingly, although OX40L-negative, all 7 of the B-NHL cell lines of different histological grade tested in the current study were found to express OX40 at higher levels compared to normal B cells. However, upon TCR stimulation in LN cell suspensions, primary malignant B cells up-regulated both OX40 and OX40L, and their growth was impaired when a neutralizing anti-OX40 mAb was added in culture. Due to the intrinsic low ability of B cells to grow *in vitro* this effect was less evident in these cells, compared to that obtained in T lymphocytes. However, this suggests the possibility that OX40-OX40L co-stimulation might contribute to tumour cell survival in a similar way of the other well-known CD40- CD40L pro-lymphoma interactions within the TNFR-TNF superfamily (Grdisa 2003; Kater, Evers et al. 2004). Whether the anti-proliferative effect on B cells is directly mediated by OX40L co-stimulation inhibition and/or depends upon the inhibition of bystander pro-survival signals remains to be ascertained. The concomitant observation that, under these conditions, FOXP3+ Treg proliferation was also impaired provides the indirect information that OX40 stimulation may promote the survival and/or the growth of regulatory lymphocytes in lymphoma. This is in agreement with previous results obtained in normal human and mouse Tregs (Kroemer, Xiao et al. 2007; Hippen, Harker-Murray et al. 2008). A stimulatory action on Tregs and tumour B cells might impair the ability of OX40 agonist reagents to effectively function as adjuvants. However, given the recognized strong activity of OX40 stimulation in promoting expansion and survival of activated lymphocytes, including anti-tumour T cells, a positive effect on these cells may be a minor issue. Furthermore, since preclinical studies in mice have indicated that OX40 triggering may transiently block Treg suppressive activity (Piconese, Valzasina et al. 2008) and peripheral conversion (Vu, Xiao et al. 2007), an increased frequency of functionally inactive Tregs should not provide much constraint to the concurrent positive immune stimulation. To assess whether this could also be true in a human setting, the use of a trimeric human recombinant OX40L fusion protein, despite showing positive co-stimulatory signals on human Teffs, revealed only a minimal capacity to counteract Treg-mediated suppression of Teff proliferation. Like other TNFR, OX40 is constitutively expressed in mouse Tregs at high levels but is inducible in the human cell counterpart (Croft 2009), indicating a more complex regulation of OX40 in human Tregs that could account for the difficulties to directly translate the findings obtained in mice to the human setting. The transient and variable expression of OX40 in both human Teffs and Tregs and the need to study its functions *in vitro* makes it difficult to understand what actually happens in Tregs rather than Teffs after OX40 stimulation in normal and pathological conditions. Ad hoc *in-vitro* assays able to accurately reproduce the *in-vivo* situation together with more effective agonist reagents are required to definitely prove the therapeutic advantages to trigger OX40 in B cell lymphoma as adjuvant treatment for anticancer immunotherapeutic approaches.

6 SUMMARY AND FUTURE PLANS

Passive immunotherapy with mAbs has dramatically improved the overall survival of patients with indolent B-NHLs (Cheson and Leonard 2008). However, no treatments are curative at the present time and the pattern of continuing relapse and progressive decrease in response to therapy requires the development of more and more effective therapeutic strategies (Gribben 2007). The possibility of counteracting the peculiar clinical course of indolent NHLs through the effective recruitment of a continuing immune response attack on tumour cells is an attractive option.

In the present study, promising results have been achieved through vaccinating heavily pre-treated relapsed indolent NHL patients with autologous DCs loaded with autologous heat-shocked, γ-irradiated, and UVC–treated tumour cells. Both T- and B-cell tumour-specific immune activation were associated with clinical responses. In particular, vaccination skewed the maturation of T cells to effector memory and/or terminally differentiated phenotype, activated NK cells, and intriguingly, reduced the frequency of Tregs in the PB and/or at tumour site. The potential of this vaccine formulation in stimulating immune responses against multiple TAAs, avoiding the selection of tumour immune escape variants, resulted biologically and clinically effective. This effect, combined with the use of DCs as carriers for the antigenic "cargo" obtained through the induction of an immunogenic tumour cell death, may provide some of the reasons for the resulting enhancement of multiple immunostimulatory signals and down-modulation of the immunoregulatory elements associated with a high objective clinical response rate (33.3%) in the vaccinated patients. Importantly, such effects were primarily induced in patients with low tumour burden, suggesting that there may be opportunities for the further optimization of vaccine strategies to eradicate disease in patients with large tumour masses.

In this pilot trial, NRs possessed lymphomas, which exhibited a decreased ability, compared to those present in Rs, to undergo to an immunogenic cell death *in vitro*. This suggests that the addition of compensatory immune stimuli within killed tumour cell-based vaccines may provide effective formulations for boosting a therapeutic anti-tumour immunity in such patients. Prospective studies examining the added value of such immune parameters as new predictors for the clinical outcome, as well as randomized trials aimed at counteracting immunologic defects, will confirm this hypothesis. Alternatively, given the growing appreciation that some conventional treatments possess the immune stimulatory property of inducing immunogenic tumour cell death (Zitvogel 2008), then they, in association with DC activating agents, may be exploited to target TAAs to DCs *in vivo*. Recently, the proof of principle that this strategy, namely *in-situ* vaccination, can be feasible, safe and potentially clinically efficacious has been provided in a pilot study in which low-grade NHL patients were treated with low-dose radiotherapy with intra-tumour injection of a TLR9 agonist (Brody, Ai et al. 2010). If confirmed, and supported by a clear biologic mechanisms in a wider series of patients, then these promising results may not only provide important information on underlying tumour immunity but, interestingly, may have the potential to pave the way for a future novel straightforward, non-customized active immunotherapy approach. Such an option will be widely applicable without the need of following clinical grade procedures for the production of the vaccine.

At present, however, active anticancer immunotherapy by either *ex-vivo* or *in-vivo* DC therapy alone has not been demonstrated to generate consistent levels of immunity capable of eradicating established diseases (Koos, Josephs et al. 2010; Claesson 2009). The strategy to combine vaccination with the transfer of tumour-competent T cells to boost tumour-specific CTL responses induced by active immunisation may increase the probability of a clinical success. The recognition of the need for CD4+ T-cell help for *in-vivo*

proliferation and activation of transferred T cells (Corthay, Skovseth et al. 2005; Perez-Diez, Joncker et al. 2007; Hunder, Wallen et al. 2008) has led to the development of expansion approaches that attempt to create a functional, endogenous, and polyclonal tumour-specific immune response of both CD8+ and CD4+ T cell subsets. The liposome-based aAPCs described in this study represent a reliable method for rapidly obtaining adequate amounts of functional and potentially long-lasting anti-tumour CTLs for anticancer ACT. The novelties of this system, which include the co-localization of T-cell ligands in artificial lipid rafts loaded on a fluid lipidic membrane and the targeting of an adhesion protein, both of which increase the efficiency of immunologic synapse formations, form the basis for its improvements over the two artificial approaches clinically available for polyclonal T-cell expansion. The use of aAPCs achieved a more efficient expansion of both polyclonal and MART-1-specific CD8+ T cells than the other systems. Stimulation with aAPCs permits for the generation of viable T cells displaying an immunophenotype consistent with the potential for their continued persistence *in-vivo* without increasing the frequency of Tregs. After stimulation with aAPCs, the specificity of anti-MART-1 CD8+ T cells was preserved and their specific cytolitic activity was significantly higher when compared to CTLs expanded using the other systems. Remarkably, the aAPC-based approach used here proved to be suitable for large-scale application minimizing the volume and the costs of T-cell expansion. The ability of this system to provide the same results when used to stimulate circulating T cells obtained from NHL patients will be matter of further studies. If this is found to be the case, then it will open up the possibility of using aAPCs for the expansion of *in-vivo* induced anti-tumour polyclonal T cells by whole tumour cell-based vaccination.

It is worth remembering that the major goal of cancer immunotherapy remains the development of a protective anti-tumour immunity that depends not only upon the amount of the induced tumour-specific effector T cells, but also, and more importantly, on their ability to persist and overcome tumour-induced immunosuppression (Koos, Josephs et al. 2010). The study of the OX40-OX40L (two members of the TNFR/TNF superfamilies) pathway in indolent lymphomas to identify novel adjuvant agents for immunotherapy thus looks very promising. Indeed, in addition to the well-known ability to boost effector T-cell function in human (Morris, Peters, et al. 2007; Croft 2009), OX40 triggering has recently been shown to abolish Treg immunosuppression in murine pre-clinical models (Valzasina, Guiducci et al. 2005; Piconese, Valzasina et al. 2008; Colombo and Piconese 2007; Vu, Xiao et al. 2007). In the present study, not only Tregs, but also their expression of OX40 was found to be increased in NHL biopsies compared to PB, thus indicating the concurrent presence of immune suppressive and inflammatory stimuli in the lymphoma microenvironment that may be crucial for the tumour growth. Accordingly, after DC-vaccination, OX40+ Tregs decreased in the PB and at the tumour site of Rs, but reappeared upon relapse in the involved LNs. OX40 stimulation by a trimeric recombinant human OX40L protein also demonstrated to boost the *in-vitro* expansion of lymphoma infiltrating Teffs in a human setting. However, this approach showed minimal effects in reversing Treg immunosuppressive functions, indicating the need of more effective agonist reagents for human OX40 and/or the identification of more suitable culture conditions to assess this activity. If OX40 stimulation demonstrates a role also in human Treg functions, then it should be possible to provide a double, apparently antithetic, activity that favours anti-tumour immunity by the use of a single agent. In addition, very preliminary results suggest that OX40-OX40L signalling may also influence malignant B cells. Indeed, under inflammatory conditions that led to OX40 and OX40L up-regulation on the lymphoma B-cell surface, OX40 neutralization by a specific Ab reduced the proliferation of primary malignant B cells *in vitro*. OX40 triggering on B-NHLs has not as yet been studied in depth. Since the axis of CD40-CD40L interaction provides crucial signals for B-cell lymphoma survival (Grdisa 2003; Kater, Evers et al. 2004), it remains important to investigate

whether OX40-OX40L co-stimulation may share similar properties. The demonstration of a direct involvement of OX40 in lymphoma cell survival will come from an accurate analysis of OX40 expression in different B-cell lymphoma histotypes coupled with the study of the functional inhibition or activation of OX40 in malignant B cell lines to reveal, respectively, more significant anti- or pro-survival effects. If this is not the case, the possibility of indirect bystander effects on lymphoma cells of OX40-OX40L co-stimulation on accessory T cells cannot be ruled out. *In-vitro* co-cultivation of lymphoma cell lines with T cells in the presence of OX40 agonist or antagonist compounds will assist in addressing this hypothesis. These studies will also be crucial to elucidate the most valuable modality of intervention through OX40-OX40L signalling in B-cell lymphoma for immune adjuvant purposes. Indeed, it is important to take into account the potential drawback of an OX40 agonist-based therapy for B-NHLs to concurrently promote the growth and/or the survival of Tregs and malignant B cells. Therefore, the possibility that the exacerbation of Teff functions by OX40 agonists at the tumour site (where inflammatory signals are precariously kept in balance for the malignant progression) may boost immunity to fight tolerance and favour cancer regression needs to be proven. Additional insights into the mechanisms whereby OX40 triggering possesses immunostimulatory properties in the human will most likely come from next results of preclinical and clinical studies with novel human OX40 agonist compounds.

The study of novel therapeutic ways for the improvement of the clinical efficacy of active immunotherapy in indolent NHL patients culminated in the extremely relevant work on the identification of HSP105 as a new potential immunotherapeutic biotarget of B-NHLs. Indeed, the induction of HSP105-specific Abs by DC-based vaccination was associated with a positive clinical outcome. The association between increasing expression levels of HSP105 and lymphoma aggressiveness and, importantly, also transformation suggests that a humoral immunity against HSP105 may keep the progression of indolent NHLs under control. This could allow the "equilibrium" phase of cancer immunoediting to be re-established. Interestingly, the blocking of HSP105 with a specific Ab significantly reduced the lymphoma burden and caused anti-vascular effects in pre-clinical murine models. This finding represents the proof of principle to consider HSP105 as another attractive target for B-NHL passive immunotherapy. The activity of this molecular chaperone in normal, as well as malignant B cells, has been poorly investigated. Like other molecular chaperones, its pro-tumour activity may be related to its properties of stabilizing oncogenes and counteracting proteotoxic stress. Interestingly, in normal rat germ cells (Kumagai, Fukuda et al. 2000) and human colorectal carcinoma cells (Hosaka, Nakatsura, et al. 2006), HSP105 has been found to stabilize and sequester p53 in the cytoplasm, a location far away from its functional nuclear site and avoiding its activation of pro-apoptotic pathway. The possibility that HSP105 chaperones p53 also in B-cell lymphoma represents an appealing hypothesis to be addressed in the near future. Loss of function of the tumour suppressor p53 is a common feature of NHLs, in particular transformed and aggressive subtypes, and leads to therapy resistance and disease recurrence (Moller, Nielsen et al. 2002; Koduru, Raju et al. 2011). Mutations of p53 have been also found to increase its protein half-life without affecting its suppressive functions. In these cases, tumours activate alternative mechanisms that can impede the re-localization of p53 into the nucleus and the induction of pro-apoptotic genes. The ability of HSP105 to stabilize p53 showed in other tissues may thus represent one of such mechanism in lymphoma. A detailed biochemical analysis of the pathways that are affected upon HSP105 inhibition, including p53, is thus required to understand the possible molecular basis for anti-HSP105 mAb-based combination therapy. Aside from a direct inhibition of pro-survival/anti-apoptotic pathways on tumour cells, the involvement of immune-mediated and anti-angiogenic effects in the promotion of the observed *in-vivo* anti-lymphoma activity of anti-HSP105 Ab needs to be studied in more details. The contribution of anti-HSP105 Ab-mediated CDC and ADCC can be analysed both

in vitro, demonstrating the dependency of the anti-tumour effects on the amounts of specie-specific serum or NK cells added in culture, and *in vivo*, by the comparison with the activity of the Ab lacking the Fc-mediated effector functions (the fragment antigen binding Fab). The "in-house" development of a specific mouse mAb, which is currently underway in collaboration with the Experimental Oncology Department of the *Fondazione IRCCS Istituto Nazionale dei Tumori di Milano*, will greatly facilitate these analyses with respect to the use of the commercially available functional polyclonal rabbit anti-human HSP105 Ab. Collectively, these studies should lead to the generation of a novel mAb, which can be transferred from the pre-clinical animal models into the clinical settings as a new passive anti-lymphoma immunotherapeutic strategy for its validation in clinical trials.

In parallel, the possibility that more specific B-cell lymphoma antigens compared to HSP105 may have induced a humoral response associated with therapeutic effects in vaccinated patients will continue to be pursued. Indeed, results obtained in this study indicate a tumour-restricted enhancement of post- versus pre-vaccine Ab reactivity in Rs. Towards this aim, in order to facilitate and potentiate the serological analyses, Ab repertoires of vaccinated patients will be investigated using protein microarrays that will permit the direct testing of serum samples from a number of patients on a very wide range of targets in a single step. Although the use of recombinant proteins on the arrays may prevent the recognition of antigens that have undergone post-translational modification, this approach has been invaluable in other studies for the detection of disease-associated autoantigens. Furthermore, the risk of losing important Ab species during Ig processing for their purification and biotin-conjugation will be reduced.

In conclusion, the work presented in the current study has fulfilled the initial aims of the project. These are as follows:

- demonstration of a correlation of a positive outcome after vaccination with the induction of anti-tumour immunity;
- the identification of potential predictors of the clinical outcome following vaccination;
- the identification of NHL-associated antigens that have the potential to be exploited as targets of passive immunotherapy;
- the identification of approaches that can be used to optimize the immune response of patients with indolent NHL following active immunotherapy.

This thesis provides important data on mechanisms underlying tumour immunity that can be used to improve and also open up new treatment options for the therapy of indolent NHLs. This information also has potential relevance for the treatment of other haematological malignancies as well as solid tumours.

7 PUBLICATIONS

7.1 Publications on the thesis project

- "Improved clinical outcome in indolent B-cell lymphoma patients vaccinated with autologous tumour cells experiencing immunogenic death."
  Zappasodi R, Pupa SM, Ghedini GC, Bongarzone I, Magni M, Cabras AD, Colombo MP, Carlo-Stella C, Gianni AM, Di Nicola M. Cancer Res. 2010 Sep 30.
- "Vaccination with autologous tumor-loaded dendritic cells induces clinical and immunological responses in indolent B cell lymphoma patients with relapsed and measurable disease: a pilot study." Blood. 2009 Jan 1;113(1):18-27.
  Di Nicola M, **Zappasodi R**, Carlo-Stella C, Mortarini R, Pupa SM, Magni M, Devizzi L, Matteucci P, Baldassari P, Ravagnani F, Cabras A, Anichini A, Gianni AM.
- "The effect of artificial antigen-presenting cells with preclustered anti-CD28/-CD3/-LFA-1 monoclonal antibodies on the induction of ex vivo expansion of functional human antitumor T cells." Haematologica. 2008 Oct;93(10):1523-34.
  Zappasodi R, Di Nicola M, Carlo-Stella C, Mortarini R, Molla A, Vegetti C, Albani S, Anichini A, Gianni AM.

7.2 Other publications during the PhD period

- "Tumor-reactive CD8+ early effector T cells identified at tumor site in primary and metastatic melanoma."
  Anichini A, Molla A, Vegetti C, Bersani I, **Zappasodi R**, Arienti F, Ravagnani F, Maurichi A, Patuzzo R, Santinami M, Pircher H, Di Nicola M, Mortarini R. Cancer Res. 2010 Sep 21.

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